J. Cell Sci. **12**, 951–981 (1973)
Printed in Great Britain

CHEMICAL AND STRUCTURAL DIFFERENCES BETWEEN CILIA AND FLAGELLA FROM THE LAMELLIBRANCH MOLLUSC, *AEQUIPECTEN IRRADIANS*

R. W. LINCK*
The Department of Biology, Brandeis University, Waltham, Massachusetts 02154, and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543, U.S.A.

SUMMARY

Gill cilia and sperm flagella from the lamellibranch mollusc *Aequipecten irradians* were isolated by several methods and chemically fractionated by low-ionic-strength dialysis. These organelles differ in their forms of dynein and in the stabilities of their homologous microtubules and subsidiary structures (i.e. nexin fibres and spoke material). In flagella more than 80 % of the axoneme ATPase is solubilized, appearing as a 14S peak in the ultracentrifuge, and the whole axoneme is broken down to *unlinked, doublet* outer fibres. In cilia only *half* of the ATPase is solubilized from axonemes as a 14S component. The ciliary B-tubules and one member of the central pair also dissolve, leaving the A-tubules and the other central tubule held together by nexin fibres and matrix material as a 9 + 1 *singlet* axoneme, to which is bound the remaining half of the ATPase. This tightly bound form of the ciliary ATPase can be removed in an enzymically active form by brief trypsin treatment which causes the breakdown of the singlet axoneme. The trypsin-solubilized ATPase behaves like native 14S dynein in the ultracentrifuge but breaks down to polypeptides when electrophoresed in the presence of sodium dodecyl sulphate (SDS).

The native 14 S material containing the dynein was partially purified by column chromatography and was shown on SDS-polyacrylamide gels to be composed of 2 components. The molecular weights of these as determined by 3 % acrylamide SDS-gels are 450000 and 500000, and they are not reduced by prior treatment with 8 M urea. Parallel SDS-gels loaded with identical amounts of ciliary and flagellar axonemes show very nearly identical components; the ratio of the amounts of the 500000 and 450000 mol. wt. components is about 2:1 respectively. The fractionation of ciliary and flagellar axonemes was analysed quantitatively on SDS-gels. Essentially all of the 450000 and 500000 mol. wt. components are found in the soluble fraction from dialysed flagella. The soluble fraction from cilia contains the 2 components in a 1:1 ratio, along with half the axoneme ATPase, while the singlet axoneme fraction contains the remainder of the 500000 material and the remaining half of the ATPase. On this basis it is believed that the 500000 component is the dynein ATPase, while the 450000 material represents a non-ATPase protein.

The chemical and structural differences between cilia and flagella may offer an explanation for their different modes of beat.

* Present address: Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England.

INTRODUCTION

The 9 + 2 microtubular axoneme with its outer fibre arms, radial spokes and circumferential linkage fibres is a remarkably constant feature of cilia and flagella throughout the plant and animal kingdoms (Allen, 1968; Gibbons, 1961; Gibbons & Grimstone, 1960; Phillips, 1970; Ringo, 1967; Warner, 1970). Although structurally similar, cilia and flagella are nevertheless functionally different (Sleigh, 1962). Cilia exert a unidirectional force on the surrounding media parallel to the cell membrane. Flagella develop a net force which is directed along the flagellar axes and away from the basal bodies. Generally speaking, movement of these organelles arises from the proximal generation of a bending region and a propagation of that bend distally (Gibbons & Gibbons, 1972; Gray, 1955; Aiello & Sleigh, 1972). Summers & Gibbons (1971) have now presented strong evidence that flagellar bending results from the sliding of adjacent outer doublet microtubules, possibly mediated by the ATPase arms.

Gibbons (1963, 1965, 1966) has fractionated and characterized the axoneme ATPase dynein and localized its site as the 2 arms on the doublet outer fibres. From *Tetrahymena* cilia 2 forms of dynein were obtained having sedimentation coefficients of 14 S and 30 S (Gibbons, 1963; Raff & Blum, 1969). The 2 dyneins differ only slightly in their enzymic properties (Gibbons, 1966) and have been related as monomer and polymer (Gibbons & Rowe, 1965). Dynein has also been obtained from sperm flagella of sea urchins (Gibbons, Fronk & Gibbons, 1970; Gibbons, 1965; Mohri, Hasegawa, Yamamoto & Murakami, 1969) and from starfish (Linck, unpublished observations), but in these cases only the 14 S form has been observed.

Tetrahymena cilia and sea-urchin sperm flagella also behave differently with regard to the linkages which maintain their 9-fold cylindrical symmetry (Gibbons, 1965). Stephens (1970*b*, 1971) has isolated the linkage protein 'nexin' and has demonstrated that these linkages interconnect the outer A-subfibres.

Differences in the forms of dynein and nexin have important consequences for a sliding filament mechanism and may offer an explanation for the different modes of ciliary and flagellar bending. In order to resolve the differences previously observed in unrelated species, cilia and flagella were compared from a single source, the lamellibranch mollusc *Aequipecten irradians*.

MATERIALS AND METHODS

Unless specified below, the materials and methods for this investigation are identical to those outlined previously (Linck, 1973).

Fractionation of ciliary and flagellar axonemes

Axonemes were suspended in *Tris-EDTA solution* (0·1 mM EDTA, 1 mM Tris, pH 8·3 at 0 °C) at protein concentrations of approximately 5–10 mg/ml and dialysed against at least 100 volumes of the same solution at 0–4 °C for 48–60 h. The dialysis solution was changed every 12 h. After dialysis the suspensions were separated into *supernatant* and *pellet* fractions by centrifugation at 35000 *g* for 10 min; dialysed flagella preparations required the presence of salt (10 mM Tris) for proper sedimentation. Protein and enzyme balances were then obtained by one of two procedures: (*a*) by direct determination from the supernatant and washed pellet

fractions, or (*b*) by calculation from values of the supernatant fraction and a sample of the whole suspension taken prior to centrifugation. Both methods provided comparable results. In some cases 0·1 mM ATP, pH 7·0, was included in the dialysis solution, as it helped to preserve the enzymic activity of flagellar dynein.

Adenosine triphosphatase activity

ATPase activity was assayed according to the procedure described in the preceding paper (Linck, 1973). The Mg^{2+}-activated ATPase was measured in the presence of 1 mM ATP, 30 mM Tris, 1 mM $MgCl_2$ and 0·1 mM EDTA, pH 8·0, at 20 °C.

SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out according to the procedures of Shapiro, Viñuela & Maizel (1967) and Weber & Osborn (1969). Pellets of protein were resuspended in *SDS-medium* containing 1 % SDS, 10 mM phosphate buffer, pH 7·0, 1 % β-mercaptoethanol, 10 % glycerol and 0·005 % bromophenol blue indicator. Solutions or suspensions of protein were diluted 1:1 with a twice-concentrated solution of *SDS-medium*. Each sample was dissolved by mixing and then heating to 100 °C for 2–5 min. An acrylamide stock solution was made by dissolving 30 g of acrylamide (Eastman-Kodak) and 0·8 of *N-N'*-methylene-bisacrylamide in deionized water and making up to 100 ml. The acrylamide gel concentration found most effective for these studies was 3 %. A typical set of gels, 80 mm long by 6 mm in diameter, was prepared from a mixture of 2·0 ml of 1·0 M phosphate buffer, pH 7·0, 0·2 ml of 10 % SDS, 2·0 ml of stock acrylamide solution, 15·6 ml of deionized water, 10 μl of TEMED (*N,N,N',N'*-tetramethylethylenediamine) and 0·2 ml of 10 % ammonium persulphate (added immediately before pouring). After pouring, the gels were layered with deionized water and polymerized at 20 °C. Electrophoresis was performed at a constant temperature of 20 °C using a water jacket, and at a constant voltage of 50 V with an electrode buffer containing 0·1 M phosphate, pH 7·0, and 0·1 % SDS. Gels were normally run until the tracking dye reached the bottom, at which time they were removed and stained for 2·5 h with 0·25 % Coomassie Brilliant Blue in 50 % methanol containing 10 % acetic acid. Gels were destained by diffusion using 10 % methanol containing acetic acid. Mobilities (R_F values) were calculated according to the following formula:

$$\text{mobility} = \frac{\text{migration distance of a given component}}{\text{migration of bovine serum albumin monomer}}.$$

Nucleotide determination

Two-millilitre aliquots of dissolved or resuspended protein of known concentration were made 5 % in trichloroacetic acid (TCA), left to stand on ice for 10 min and then spun at top speed in a clinical centrifuge for 3 min. The supernatants were withdrawn and the tubes and pellets washed with 3 drops of deionized water; the rinses were added to the supernatants. Nucleotide standards and a water sample were also made 5 % in TCA. All samples were extracted 10 times with 3 vol. of water-saturated diethyl ether at 0 °C. Optical density spectra (220–360 nm) of the samples were run against the ether-extracted water blank, using a Beckman double beam spectrophotometer and a hydrogen lamp. The samples were then lyophilized in conical centrifuge tubes and stored at −20 °C for later use. For chromatography each sample was dissolved in 50 μl of deionized water and spotted on an Eastman-Kodak cellulose thin layer plate (no. 6064). Ascending chromatography was carried out using an isobutyric acid, concentrated ammonia and distilled water solvent in a ratio of 57:4:39 respectively at pH 4·3. Following air-drying, the plates were examined with a 254-nm ultraviolet lamp.

Concentration of protein solutions

Concentrated protein solutions for column chromatography or for use in the analytical ultracentrifuge were prepared either by absorption through a dialysis membrane using dry Sephadex G-100 or by ultrafiltration using an Amicon 'Diaflo' Ultrafiltration Cell and a UM-20E filter, operated at 275·8 kN m^{-2} of compressed nitrogen. Both methods were carried out at 0–4 °C and gave comparable results.

Sephadex gel filtration

Chromatography of protein concentrates was carried out using a Pharmacia reverse flow apparatus and a Sephadex G-200 gel bed, equilibrated with 1 mM Tris, 0·1 mM EDTA, pH 8·3, at 0 °C. A pressure head of 100 mm was used to maintain a constant flow rate of about 0·3 ml/min. A solution of Blue Dextran 2000 was used to determine the void volume. Half-millilitre fractions were collected from a 25 × 300 mm gel column.

Analytical ultracentrifugation

Sedimentation velocity runs were performed on a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics, a phase plate and a temperature control. Photographs were taken on Kodak Metallographic plates. The plates were measured on a Nikon Model 6 microcomparator, and the results were evaluated and corrected to standard conditions using standard methods (Schachman, 1959).

Electron microscopy

Thin sections were prepared as described in the preceding paper (Linck, 1973). Negatively stained preparations were made with 1% uranyl acetate according to the procedures of Brenner & Horne (1959) and Huxley (1963). Shadow casting was performed according to the method of Hall (1960) by spraying protein samples on to freshly cleaved mica, shadowing with platinum and coating with a carbon film. Specimens were floated on to a water surface and picked up with 400-mesh grids.

Observations were made and photographs were taken on a Philips 300 electron microscope fitted with a 30-μm objective aperture and operated at 60 kV. Calibration of the magnification taps was based on the 39·5–40·0 nm spacing of Mg^{2+}-tactoids of rabbit tropomyosin provided by Dr Andrew G. Szent-Györgyi (Caspar, Cohen & Longley, 1969).

RESULTS

Fractionation of ciliary and flagellar dyneins

Ciliary and flagellar axonemes from *Aequipecten irradians* were fractionated by low ionic strength dialysis according to the procedure of Gibbons (1963) and were found to differ in a number of ways as summarized in Fig. 1.

Dialysed axonemes were separated into supernatant and pellet fractions by high-speed centrifugation; each fraction was then assayed for its total protein and ATPase content. The results of these fractionation procedures are given in Tables 1 and 2. Extensive dialysis solubilizes 30–35% of the ciliary and flagellar axoneme protein, and in doing so slightly activates the axoneme ATPase (see '% activation of enzyme units' in Tables 1, 2). For example, after dialysis the enzyme units (μmol P_i/min) of ciliary and flagellar axonemes were increased to $127 \pm 26\%$ (Table 1A) and $147 \pm 20\%$ (Table 2) of their original levels respectively. Of the enzyme units remaining after dialysis, more than 80% was extracted from flagellar axonemes, as compared with only 40–50% from ciliary axonemes. For cilia, the actual percentage of soluble enzyme units varies according to the method of cilia isolation: $43 \pm 8\%$ for cilia isolated from twice-concentrated seawater (based on 17 measurements); $51 \pm 6\%$ for cilia prepared from 10% ethanol–10 mM $CaCl_2$ (5 measurements); and 65% for cilia isolated with 60% glycerol (1 measurement). In spite of this variation, the percentages for the 3 different procedures are *all* significantly smaller than the percentage of dynein

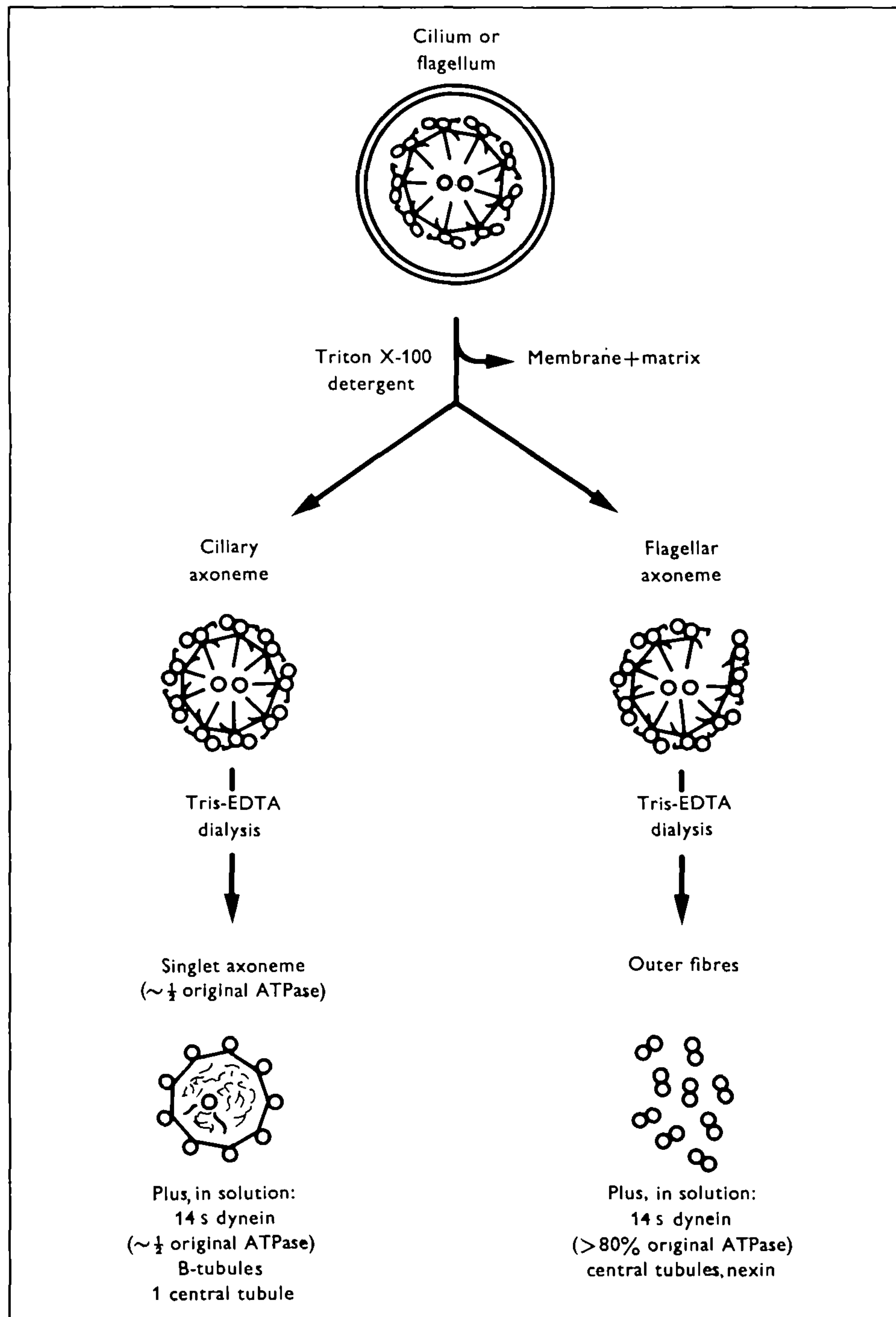


Fig. 1. A schematic diagram illustrating the differential fractionation of gill cilia and sperm flagella from *Aequipecten irradians*.

Table 1. *Fractionation of A. irradians ciliary axonemes by dialysis*

Dialysis solution	Initial specific activity of axonemes	Activation of EU's, %	Supernatant fraction: Protein, %	Supernatant fraction: EU's, %	Pellet fraction (singlet axoneme): Protein, %	Pellet fraction (singlet axoneme): EU's, %	No. of experiments
	A, Results obtained from cilia isolated from 2 × seawater						
Tris-EDTA	0·18 ± 0·05	124 ± 27	31 ± 3	44 ± 6	69 ± 3	56 ± 6	13
Tris-EDTA-ATP	0·16 ± 0·02	137 ± 20	28 ± 1	40 ± 12	72 ± 1	60 ± 12	4
Average	**0·17 ± 0·04**	**127 ± 26**	**30 + 3**	**43 ± 8**	**70 ± 3**	**57 ± 8**	17
	B, Results obtained from cilia isolated from 10 % ethanol–10 mM $CaCl_2$						
Tris-EDTA-ATP	0·13 ± 0·02	133 ± 6	29 ± 5	51 ± 6	71 ± 5	49 ± 6	4
	C, Results obtained from cilia isolated from 60 % glycerol						
Tris-EDTA-ATP	0·12	157	37	65	63	35	1

NOTATIONS. *Tris-EDTA(-ATP)*, 1 mM Tris, pH 8·3, 4 °C, 0·1 mM EDTA (0·1 mM ATP); initial specific activity in μmol P_i/min/mg protein; EU's, enzyme units in μmol P_i/min; % activation of EU's (EU's after dialysis ÷ EU's before dialysis) × 100; % EU's in supernatant and pellet fractions are based on the total EU's remaining after dialysis.

Table 2. *Fractionation of A. irradians flagellar axonemes*

Dialysis solution	Initial specific activity of axonemes	Activation of EU's, %	Supernatant fraction: Protein, %	Supernatant fraction: EU's, %	Pellet fraction (outer fibres): Protein, %	Pellet fraction (outer fibres): EU's, %	No. of experiments
Tris-EDTA-ATP	0·12 ± 0·06	147 ± 20	35 ± 5	82 ± 2	65 ± 5	18 ± 2	4

NOTATIONS. As in Table 1.

extracted from flagella. On this basis it is believed that the dynein-solubility differences between cilia and flagella are real and are not the result of the isolation procedures.

It has recently been shown (Gibbons & Gibbons, 1972; Gibbons & Fronk, 1972) that the properties of bound and soluble dynein are affected differently by conditions of salt and pH. Thus the apparent activation of *Aequipecten* dynein upon dialysis can be explained in terms of the conditions used for the ATPase assay. In the present investigation, ATPase activities were assayed under conditions similar to those of Gibbons & Fronk (i.e. near the point of intersection in their fig. 5) in which dynein is only *slightly* activated upon extraction; thus the percent of enzyme units in the dialysis supernatant (Tables 1, 2) remains an accurate estimate of the amount of dynein solubilized.

Ultrastructural studies on fractionated cilia and flagella

Since ciliary and flagellar axonemes differ in their amounts of extractable ATPase, it was hoped that electron-microscope studies on the fractionated organelles would reveal the site or sites of the 2 forms of the ATPase in ciliary axonemes. Unexpectedly, these observations revealed a number of other interesting structural differences between cilia and flagella, schematically illustrated in Fig. 1.

Following removal of the membrane with 1 % Triton X-100, flagellar axonemes of *Aequipecten* appear as fragmented arrays of the 9+2 structure, due to partial solubilization of the nexin fibres (Linck, 1973). After extensive dialysis of the already partially opened flagellar axonemes, all of the secondary components are solubilized, i.e. the nexin and radial linkages. Cross-sections (Fig. 3A) and negatively stained preparations (Fig. 3C) indicate that only unassociated doublet outer fibres remain. After only 24 h of dialysis (Fig. 3B) the outer fibre doublets are still associated in groups of 2 and 3 via the nexin fibres. The fundamental repeat of the nexin linkages is approximately 95 nm; however, their variable width in negatively stained preparations makes it difficult to determine their precise organization along the outer fibres.

Aequipecten cilia behave quite differently upon fractionation. Following a 48-h dialysis, ciliary axonemes break down into cylindrically shaped, 9+1 'singlet axonemes' (Fig. 4), consisting of the 9 outer A-subfibres, an amorphous matrix and usually 1 remaining central microtubule. After further dialysis the remaining central fibres are solubilized, leaving only the cylinder of A-subfibres and matrix material. Arms are not observed on the singlet A-subfibres, nor were they readily seen on A-subfibres of whole ciliary or flagellar axonemes (Linck, 1973). The A-subfibres can be distinguished from the less stable B-subfibres in partially fractionated outer fibre complexes (Fig. 4B, G), where skewing of a few remaining outer fibre doublets permits identification. The dense projections that are occasionally observed on A-subfibres are inner wall remnants of the B-subfibres, rather than inner arms. Fig. 4H illustrates the typical manner in which B-subfibres are solubilized, i.e. beginning at one junction of the A-subfibre and ending at the other junction as a dense granule that protrudes into the lumen of the A-subfibre.

Structures such as the nexin and radial linkage fibres are difficult to observe in cross-sections of dialysed ciliary axonemes, but the remarkably constant cylindrical arrangement of the A-subfibres attests to the presence of one or both of these components. The matrix region of singlet axonemes still contains a substantial amount of electron-dense material, and in one case (Fig. 4G and diagrammed in Fig. 4H) fibrous structures resembling the spokes are seen radiating sinuously outward from the centre to the A-subfibres. Matrix material resembling spokes is also observed in longitudinal sections of ciliary singlet axonemes, as in Fig. 5C.

It was thought that the remaining half of the ciliary ATPase bound to the singlet axoneme might have become disorganized during dialysis, and that its original location might be observed by redialysing the singlet axonemes under the conditions used for the reconstitution of 30S dynein (Gibbons, 1965), namely 30 mM Tris, 3 mM $MgCl_2$,

0·1 mM EDTA, pH 8·3, at 0–4 °C. The results of the attempted reconstitution indicate, however, that no appreciable reorganization of arms or spokes takes place.

Unlike dialysed flagellar outer fibres which are no longer connected by nexin linkages, the ciliary counterparts remain intact, appearing as tightly applied fibre bundles in negatively stained preparations (Fig. 5A). Occasionally ciliary singlet axonemes fray apart (Fig. 6) in such a way that individual singlet A-subfibres and the associated nexin can be seen. As in the case of partially fractionated flagellar axonemes, the fundamental repeat of the ciliary nexin fibres is estimated at 95 nm.

The possibility exists that the solubilization of flagellar nexin after dialysis results from small amounts of acrosomal proteases contaminating the preparation. Such a possibility is believed to be unlikely, since detached flagella are easily separated from the intact sperm heads and since preparations of axonemes are washed repeatedly with Triton and buffer solutions at 0 °C. Isolated cilia, on the other hand, may experience more protease action during lysis of the gill epithelia, but the nexin remains intact on dialysis. The intact membranes of the isolated organelles are probably effective barriers to proteolytic activity.

The basal ends of isolated ciliary axonemes and fractionated outer fibre complexes are held together by densely staining collar regions, 0·25–0·30 μm in length, which correspond in cross-section to the transition zones of cilia (see Gibbons & Grimstone, 1960). In negatively stained preparations (Fig. 5A) the transition zone is a bulbous structure, slightly larger in diameter than the singlet axoneme – about 0·17 μm at its widest point. In longitudinal sections (Fig. 5B, C) of singlet axonemes the bulbous shape is explained by a region of densely staining doublet outer fibres extending the length of the transition zone. At the extreme end the outer fibres pass into a dense basal plate containing an even more electron-dense core. The negatively stained singlet axoneme in Fig. 6B emphasizes the apparent fusion that the transition zone makes with the A-subfibres, all of which are frayed apart except at their base.

Identification and molecular-weight determination of ciliary and flagellar dyneins

The supernatants from the dialysis of ciliary and flagellar axonemes were concentrated and then examined in the ultracentrifuge. Both ciliary and flagellar supernatant fractions contain 4S and 10S components measured in 10 mM Tris, 0·1 mM EDTA, pH 8·0, at 20 °C (Fig. 7A, B). In neither case is a faster peak observed (e.g. 30 S). The sedimentation coefficients increase to 6 S and 14 S in 0·1 M KCl and correspond to the 6 S tubulin dimer and the 14 S dynein respectively, obtained from *Tetrahymena* cilia (Gibbons, 1963); hereafter the slow and fast sedimenting species will be referred to as the 6 S and 14 S components.

Aequipecten ciliary dynein from concentrates of the supernatant fraction has been partially purified by column chromatography using Sephadex G-200 eluted with Tris-EDTA or Tris-EDTA-ATP. Dynein, with a molecular weight of at least 400000 was collected in the void volume, followed closely by a tubulin dimer (120000 mol. wt.). The ATPase activity of ciliary dynein was increased from 0·31 to 1·21 μmol P_i/min/mg protein, and the *two* high-molecular-weight bands on SDS-polyacrylamide gels were greatly enhanced (Fig. 8F). Shadow-cast preparations indicated a major

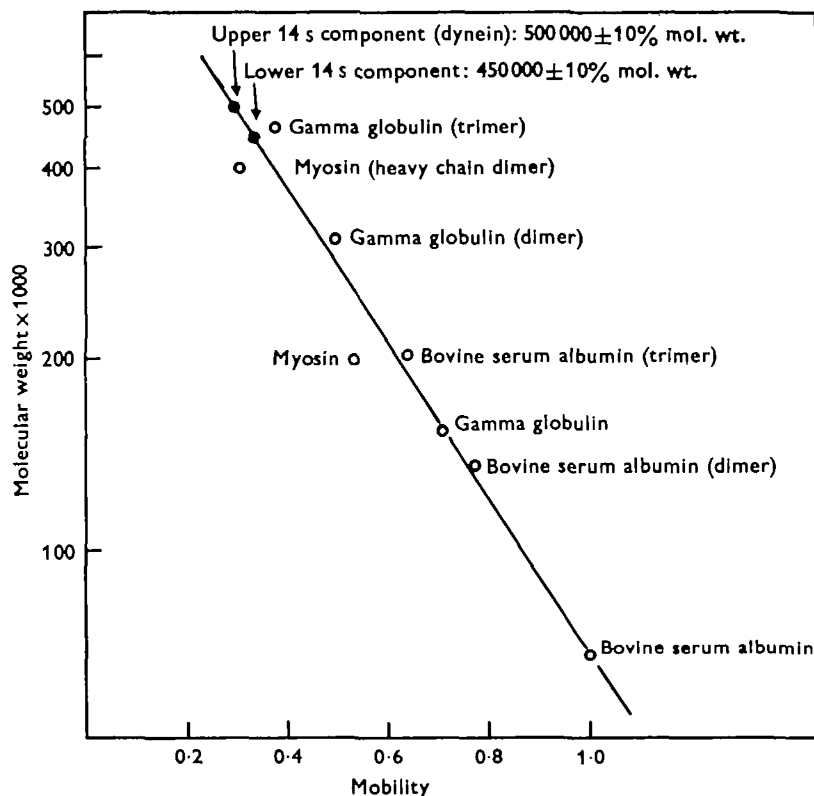


Fig. 2. Plot of molecular weight vs. mobility on SDS-polyacrylamide gels (3 %). The plot is based on gels A–E of Fig. 8. Solid circles denote the 2 components of 14 S dynein. Open circles designate mol. wt. standards: bovine serum albumin (68000); gamma globulin (155000); rabbit myosin, heavy chain monomer (200000).

component consisting of a globular protein approximately 10 nm in diameter, and indistinguishable from those of Gibbons (1968*a*, fig. 9).

Molecular weights of the 2 components occurring in preparations of ciliary and flagellar dynein have been estimated using SDS-polyacrylamide gel electrophoresis, according to the methods of Shapiro *et al.* (1967) and Weber & Osborn (1969). A set of standards and samples of ciliary and flagellar dynein were electrophoresed on 3 % gels (Fig. 8 A–E), and the mobilities of the standards plotted against the logarithms of their molecular weights. The graph obtained (Fig. 2) was linear over a molecular weight range from 68000 to 500000. Two independent determinations were made. The molecular weights estimated for the upper and lower bands are 500000 ± 10 % and 450000 ± 10 % respectively. There are no detectable molecular-weight differences in these 2 components for cilia and flagella from *Aequipecten* or for flagella from several species of sea urchins, including *Arbacia punctulata* and *Strongylocentrotus droebachiensis*. A preliminary investigation (Linck, 1970) reported molecular-weight values of 290000 and 260000 for the 2 bands, based on 5 % acrylamide SDS-gels; on closer examination of the 5 % system, however, the molecular-weight plot was found to be

non-linear above 300000 mol. wt. The ciliary and flagellar 14 S materials (i.e. the supernatant fraction from dialysis) were treated with 8 M urea for 2–24 h before preparing them for SDS-gel electrophoresis. This application, however, did not reduce the molecular weights of the two 14 S components (Fig. 8 G).

Analysis of whole fractionated axonemes by SDS-polyacrylamide gel electrophoresis

Whole and fractionated ciliary and flagellar axonemes of *Aequipecten* and also flagellar axonemes of the sea urchin *S. droebachiensis* were compared by SDS-polyacrylamide gel electrophoresis (Fig. 10). The general patterns of both ciliary and flagellar axonemes are similar: (1) a principal broad band (labelled *t*) near the bottom, corresponding to the 60000 molecular-weight tubulin monomer; (2) a pair of sharp, closely spaced bands (labelled *d*) of high molecular weight representing the 14 S material; and (3) a series of lighter secondary bands in between. Whereas all of the flagellar proteins migrate on a 3 % gel, ciliary axonemes contain a component which is not broken down in 1 % sodium dodecyl sulphate-1 % mercaptoethanol and which sits as a crust on the top surface of the gel (Fig. 10 A, B). Microdensitometry of the high-molecular-weight double bands is difficult owing to their proximity, but by inspection it appears that the upper, heavier component is approximately twice as dense as the lower one and that ciliary and flagellar axonemes contain similar amounts of protein in these bands per unit weight of axoneme (Fig. 10 A–G).

The supernatant and pellet fractions from dialysis have been analysed quantitatively by placing stoichiometric amounts of each on gels, so that their sum equals the amounts placed on gels of whole axonemes (Fig. 10 H–O). The ciliary supernatant (Fig. 10 I) contains both of the higher-molecular-weight doublet bands in roughly equal amounts, whereas the ciliary pellet, which still possesses half of the axoneme ATPase, contains only the upper band, comparable in density to the upper band of the supernatant (Fig. 10 J). Essentially all of the original double bands appear in the supernatant of *Aequipecten* flagella, but in the pellet (Fig. 10 L) only a trace of the upper band remains. A parallel fractionation of sea-urchin flagellar axonemes from *S. droebachiensis*, in which 90 % of the ATPase has been solubilized, resembles the fractionation of *Aequipecten* flagella (see Fig. 10 M–O). With *Aequipecten* flagella, less of the 60000 molecular weight component (tubulin) and more of the secondary bands (e.g. nexin and spoke material) occur in the flagellar supernatant than in the pellet (cf. Stephens, 1970*b*), as is expected, since outer fibre doublets remain intact and nexin fibres and spokes dissolve. In contrast, the ciliary supernatant contains nearly as much tubulin (B-subfibres and one central fibre) as the pellet (A-subfibres and one central fibre) but almost none of the secondary bands, while the pellet consisting of the outer fibre complexes retains the SDS-insoluble material which layers the top of the gel.

Subfractionation of ciliary outer fibre complexes

Efforts were made to isolate the bound form of the ATPase from the dialysed ciliary axonemes. Extraction of the dialysis pellet with 1 M KCl or 0·5 M KI failed to solubilize the remaining ATPase or to remove the bound 500000 component. Certain other

attempts to fractionate this ATPase were also unsuccessful: sonication for 1 min; thermal treatment in 1 mM Tris, pH 8·0 at 40 °C for 2–10 min; extraction with 0·5 M Na_2CO_3, pH 10·5; and low ionic strength dialysis under more acidic (pH 6·5) and more alkaline (pH 9·5) conditions. This insoluble form of the ATPase can be released, however, by brief trypsinization of the singlet axonemes. The trypsin treatment (according to the method of Szent-Györgyi, 1953) was carried out at 20 °C using a trypsin-to-axoneme ratio of 1:300 (w/w) and was stopped by the addition of excess trypsin inhibitor. Such a treatment causes the immediate release of two thirds of the remaining ATPase, and following a subsequent 12-h Tris-EDTA dialysis of the trypsinized singlet axonemes, all of the ATPase and outer fibres are solubilized. In the analytical ultracentrifuge the solution shows the familiar 3 and 9 S peaks in Tris-EDTA (Fig. 7C). On 3% SDS-polyacrylamide gels (Fig. 9B, C) the tubulin and secondary bands migrate with their usual mobilities, but the SDS-insoluble material which formerly incrusted the gel has migrated into the gel surface. Also, no high-molecular-weight dynein band is observed, suggesting that the trypsin-released dynein has been degraded in SDS polypeptides. It was also noted in these studies that the brief trypsin treatment used to solubilize the tightly bound ciliary dynein causes a 2-fold activation of the ATPase activities of both intact axonemes and dialysed singlet axonemes.

Dialysed ciliary axonemes were also examined under denaturing conditions. Following twice-repeated extractions with 1–2% Sarkosyl (sodium lauryl sarcosinate), 10 mM Tris, pH 8·0, at 20 °C, roughly 10% of the protein remains undissolved and can be removed by centrifugation at 48000 **g** for 15 min. Under these conditions the ATPase activity is completely and irreversibly destroyed. On SDS gels (Fig. 9E) this sedimentable material corresponds to that which does not penetrate the gel surface, along with some residual tubulin; most of the tubulin and secondary bands and the remainder of the upper dynein bands appear in the gel of the Sarkosyl extract (Fig. 9D). Negatively stained and shadow-cast preparations indicate that the insoluble material consists of the basal ends (transition zones) of the cilia, along with some associated, badly fragmented, singlet outer fibres.

Chemical studies on homologous microtubules of cilia and flagella

Bound guanine nucleotides were examined as a possible basis for the solubility differences of the various ciliary and flagellar microtubules outlined above. Ciliary A- and B-subfibres were fractionated by dialysis and were compared with similarly prepared outer fibre doublets from *A. irradians* and *S. droebachiensis* sperm flagella. The ultraviolet absorption spectra of the nucleotides were compared with a GTP standard, following the TCA treatment and ether extraction of the TCA supernatant. The samples and a series of standards including guanine, GMP, GDP and GTP were chromatographed on thin layer cellulose plates. Before dialysis ciliary and flagellar axonemes contained equivalent amounts of guanine nucleotide. After fractionation, all of the tubulins examined contained bound guanine nucleotide in the di- and triphosphate form. The ciliary B-tubulin, however, contained substantially less nucleotide per mg of protein than the A-tubulin, suggesting that the nucleotide was lost on solubilization of the B-subfibre.

DISCUSSION

Differences in ciliary and flagellar dyneins

Both inter- and intraspecies comparisons of cilia and flagella indicate that these organelles may differ chemically according to their dyneins, microtubules and subsidiary structures. Both 14 and 30 S forms of dynein have been obtained from *Tetrahymena* ciliary axonemes following low ionic strength dialysis or extraction with 0·6 M KCl (Gibbons, 1965), or by extraction with 20 mM ATP (Raff & Blum, 1969). On the other hand, all of the ATPase is extracted as a 14 S component from sperm flagellar axonemes of sea urchins (Gibbons & Fronk, 1972; Gibbons *et al.* 1970; Gibbons, 1965; Mohri *et al.* 1969) and the starfish *Asterias* (Linck, unpublished observations). The present investigation, schematically illustrated in Fig. 1 (p. 955), has demonstrated that more than 80% of the axoneme ATPase can be removed from molluscan sperm flagella as a 14 S component by low ionic strength dialysis, whereas in gill cilia of this species half of the ATPase is obtained in the 14 S form and half remains tightly bound to or confined by the singlet axoneme. Mohri (1964) previously reported that in fish sperm of *Prionotus* only half of the ATPase activity could be removed from flagellar axonemes by low ionic strength dialysis or salt extraction. With the possible exception of *Prionotus* then, flagellar dynein is solubilized as a 14 S component, whereas ciliary dynein is obtained in both 14 S and *associated* forms.

Similarities among ciliary dyneins

From physical-chemical and enzymic data, Gibbons & Rowe (1965) related 14 and 30 S dyneins in *Tetrahymena* as monomer and polymer respectively, suggesting that the former was a partial breakdown product of the latter. Some variability has been found in the relative amounts of the 2 forms, but in general Gibbons (1965) found a weight ratio of 3:8 for 14–30 S, and Raff & Blum (1969) reported a ratio of 4:9. Using the specific activities of 3·5 and 1·3 μmol P_i/minute/mg protein obtained by Gibbons (1966) for purified 14 and 30 S dyneins respectively, one can compare the actual enzyme units of the 2 forms. For the 3:8 weight ratio above, the 14 and 30 S dyneins each represent 50% of the total axoneme ATPase activity (μmol P_i/min). With the gill cilia from *Aequipecten* the situation is much the same. The percentage of enzyme units remaining bound to the axoneme after dialysis varies somewhat with the cilia isolation procedure: 57 ± 8% for cilia isolated from twice-concentrated seawater; 49 ± 6% for cilia from the ethanol/calcium procedure; and 35% for glycerol-isolated cilia (see Table 1). Since the activities of the axoneme ATPase are enhanced after dialysis, the variation in the proportion of the soluble and tightly bound forms of dynein may result from either a preferential activation of one form or, if both forms are similarly activated, a conversion (e.g. breakdown) of one form to the other.

Monomeric dyneins from *Aequipecten* and *Tetrahymena* have comparable sedimentation coefficients and molecular weights (see below) and both appear as 10-nm globules in shadow-cast preparations. The polymeric 30 S dynein from *Tetrahymena* is

broken down by brief trypsin treatment to yield products with 14 S dynein-like physical and enzymic properties (Gibbons, 1966, 1968*b*): in particular, trypsin treatment causes a rise in the ATPase activity of 30 S dynein toward that of 14 S dynein. Similarly in *Aequipecten* gill cilia the tightly bound ATPase is solubilized in an enzymically active state by a brief trypsin treatment, after which 3 and 9 S peaks appear in the ultracentrifuge as compared with the 4 and 10 S components of the ciliary dialysis supernatant fraction in a similar solvent (Fig. 7). Like 30 S dynein, the tightly bound ATPase undergoes a 2-fold activation following trypsin treatment.

The multicomponent nature of 14 S *dynein*

The molecular weight of purified 14 S dynein from *Tetrahymena* has previously been estimated at 600000 ± 100000, using sedimentation equilibrium (Gibbons & Rowe, 1965). The SDS-acrylamide gel technique now indicates that ciliary and flagellar 14 S material from the dialysis supernatant fraction actually consists of 2 components with molecular weights of 450000 ± 10% and 500000 ± 10% (Figs. 2, 8). On SDS-gels of both ciliary and flagellar axonemes (Fig. 10A–G) the double bands exist in a density ratio of approximately 2:1, upper to lower. SDS-gels of the soluble fraction from cilia contain the 2 bands in approximately equal proportion; whereas gels of the ciliary pellet fraction, which still possesses half of the original axoneme ATPase, contain only 1 of the 2 bands, corresponding in density and position to the higher 500000 component (Fig. 10 H–J). In contrast to cilia, the flagellar supernatant fraction, with more than 80% of the axoneme ATPase, contains essentially all of the 500000 and 450000 components in a 2:1 ratio (Fig. 10K–O). Since the upper band remains in dialysed ciliary axonemes, and since this fraction contains half of the original ATPase, it is suggested that this upper 500000 component is the dynein ATPase, and that the lower 450000 molecular weight protein represents a non-ATPase, perhaps necessary for modifying half of the dynein or for binding it to a specific site on the axoneme.

It is relevant to point out here that in the sea-urchin embryo, the higher-molecular-weight band is present prior to fertilization and that during development up to the blastula stage only the lower-molecular-weight component is synthesized (Stephens, 1972). Also, Mazia, Chaffee & Iverson (1961) and Weisenberg & Taylor (1968) have described a 13 S dynein-like ATPase in sea-urchin eggs. It is an attractive hypothesis to suppose that the 500000 mol. wt. component is present prior to ciliogenesis and that during ciliogenesis the 450000 mol. wt. protein is synthesized which participates in the assembly of dynein on the ciliary axoneme.

It is also interesting that in preparations of purified intact microtubules from pig brains, high-molecular-weight proteins have been demonstrated on SDS-polyacrylamide gels (Kirkpatrick, Hyams, Thomas & Howley, 1970). The authors have suggested that these components may be the 'arms' frequently associated with microtubular systems. The appearance of these high-molecular-weight bands on SDS-gels is strikingly similar to that of ciliary and flagellar dynein.

Gibbons (1967) reported that the molecular weight of *Tetrahymena* 14 S dynein is reduced in 5 M guanidinium-HCl to 220000 and some lower-molecular-weight material, accurate values being limited by heterogeneity and aggregation. In *Aequi-*

pecten on the other hand, the molecular weights of the two 14 S components are unaffected by 8 M urea (Fig. 8). *Tetrahymena* 30 S dynein can be broken down to 13 and 11 S components by treatment with 0·1 M Na_2CO_3 (Gibbons, 1963). In contrast again, the molecular weight of the tightly bound *Aequipecten* dynein is not reduced by similar alkaline conditions. The significance of these differences between *Aequipecten* and *Tetrahymena* dyneins should become obvious when the latter are analysed using the SDS-polyacrylamide gel technique.

Localization of the 14 S *components*

An important question raised by this investigation concerns the localization of these two 14 S components. Gibbons (1965) has demonstrated that purified 30 S dynein can be reconstituted with ciliary outer fibres, reappearing as the arms on the A-subfibres; substantially less reconstitution occurs with 14 S dynein. In *Aequipecten* the arms are not readily apparent on enzymically active ciliary and flagellar axonemes (see Linck, 1973); however, the difficulty with which arms are observed has been interpreted as a loss of electron density due to random orientations of the arm structures. If in fact the arms are present in *Aequipecten* ciliary and flagellar axonemes, and if the arms represent the only locus of the axoneme ATPase, then the macromolecular heterogeneity of the ciliary 14 S dynein and the homogeneity of the tightly bound dynein might offer some explanation for the occurrence of the two *asymmetric* arms and/or the multi-subunit appearance of the outer, more hooked arm (Allen, 1968). For example, the outer arm might be composed of the 2 high-molecular-weight components, one an ATPase and the other a structural subunit necessary for bonding half of the ATPase to a specific site on the tubule; the inner arm might then consist of only the ATPase component, strongly bonded to the tubule either directly or via the lower molecular-weight material. Gibbons & Fronk (1972) have recently demonstrated that salt extraction of flagellar axonemes from sea-urchin sperm solubilizes 50–70% of the dynein and preferentially removes the *outer* arms. This evidence supports the concept of 2 structurally and chemically different dynein arms. If this is the case, then the inability of the native 14 S dynein to reassociate may be a function of the more complex association of the multi-subunit outer arms, while the reassociation of the 30 S form might be less specific, allowing for the reappearance of both inner and outer arms (Gibbons, 1965). The necessary use of trypsin to release an active form of the tightly bound ATPase from singlet axonemes, or of Sarkosyl to release the same in a denatured state, may also suggest ways in which this dynein is bound by the axoneme: (1) it may be strongly bonded to a single protofilament of the A-subfibres; or (2) it may exist as a polymer (e.g. of inner arms) which are randomly coiled within the singlet axoneme and physically confined by the cylindrical net of closely spaced nexin linkages and A-subfibres.

One other possibility exists for the location of dynein other than or in addition to the A-subfibre arms. The singlet ciliary axonemes, which contain half of the total ATPase after dialysis, also possess a significant amount of amorphous material in the matrix region (see Fig. 4). The matrix region of intact organelles is largely composed of radial spokes, i.e. radial link heads and radial link fibres (Hopkins, 1970; Warner, 1970),

and these structures seem to be present but disorganized in dialysed ciliary singlet axonemes from *Aequipecten* (Figs. 4G, H, 5C). In cross-section, the radial link heads of *Aequipecten* measure approximately 10 nm in diameter (fig. 2 of Linck, 1973). Their globular shape and size corresponds roughly to a molecular weight of 500000 and their location within the axoneme is a suitable one (as is that of the A-subfibre arms) for a sliding filament mechanism of motility; only in this case sliding would take place between the central structure and the cylinder of outer fibre doublets. Since only the 30 S dynein of *Tetrahymena* reconstitutes as the arms of the A-subfibres, perhaps the ciliary 14 S dynein (or half of it in flagella) has a different location – the link heads – the reconstitution of which would be dependent on a preassembled central structure.

Differences in ciliary and flagellar microtubules

It has been known for some time that microtubules differ in their susceptibility to heat, cold and enzymic digestion (Behnke & Forer, 1967). A-subfibres are most stable, followed by B-subfibres, central pair and accessory tubules and finally cytoplasmic microtubules. The studies on *Aequipecten* provide an example in which even corresponding ciliary and flagellar microtubules differ in their solubilities. Flagellar outer doublet microtubules are about as stable as ciliary A-subfibres during low-ionic-strength dialysis; however, the flagellar B-subfibres can be thermally fractionated according to the method of Stephens (1970*a*) for sea-urchin sperm flagellar outer fibres. One of the ciliary central pair microtubules is less stable than the other and corresponds in its solubility to the ciliary B-subfibres and the flagellar central pair. The more stable of the central fibres is termed the C_2-tubule after Jacobs, Hopkins & Randall (1968) and Jacobs & McVittie (1970), who find a similarly more labile central tubule in *Chlamydomonas* flagella. The C_1-tubule of *Aequipecten* cilia also dissolves, but only after extensive dialysis. Thus the ciliary and flagellar microtubules can be ranked in decreasing order of their stabilities to low-ionic strength dialysis and thermal fractionation: (1) ciliary and flagellar A-subfibres; (2) flagellar B-subfibres; (3) one member of the ciliary central pair (the C_1-tubule); and (4) ciliary B-subfibres, the other member of the ciliary central pair (the C_2-tubule) and both members of the flagellar central pair. The microtubule differences illustrated here would seem to be related to actual chemical differences within the tubulin proteins. Considering the greater stability of the flagellar outer fibre doublets in the total absence of secondary structures (nexin and radial linkages), in contrast to the lability of ciliary B-subfibres in the continued presence of such structures, it is difficult to conceive how these secondary structures could be important factors controlling tubule stability. In view of the recent suggestion that microtubules are composed of heterodimers (Bryan & Wilson, 1971; Fine, 1971; Feit, Slusarek & Shelanski, 1971; Olmsted, Witman, Carlson & Rosenbaum, 1971; Witman, 1970), there are 2 possible explanations for the chemical differences in microtubules from *Aequipecten*: either the organism is capable of modifying its tubulin monomer pool once synthesized, or its genome is capable of producing a number of different, non-allelic tubulin monomers (Stephens, 1970*a*). In either case a cell could construct the desired microtubule from an appropriate combination of monomer types. Regardless of how the different tubules are

assembled, the question remaining is how such differences relate to the structure and/or function of cilia and flagella. For example, the ciliary and flagellar B-subfibres may be responsible for determining the precise lengths to which the outer fibre doublets polymerize in the respective organelles (sperm flagella are twice as long as gill cilia); on the other hand the B-subfibres could be directly involved in the mechanochemical process and thus affect the beating patterns of cilia and flagella.

Mechanochemical considerations of ciliary and flagellar bending

Satir (1965, 1967) studied the cross-sectional patterns of outer doublet and singlet fibres in the tips of effective, recovery and resting cilia and provided the first indirect evidence for a sliding-filament mechanism, wherein a given outer fibre doublet slides up to 5% of its length relative to the furthermost member in the 9+2 axoneme. Whether the sliding was an active or passive process, however, could not be determined. Summers & Gibbons (1971) have more recently presented evidence that outer doublet microtubules of flagella slide, possibly by means of dynein arms. The parameters for the reactivation of sea-urchin sperm have been thoroughly investigated (Gibbons & Gibbons, 1972), as have the properties of bound and soluble dynein (Gibbons & Fronk, 1972). A crucial question is how such sliding of microtubules can be converted into a bending wave, and related to this is the question of how bending waves are modified to produce different modes of beating in cilia and flagella. The differences and similarities between ciliary and flagellar dyneins have been discussed above. Considering the function of dynein as an ATPase which presumably interacts with the microtubules, any differences in dynein properties could easily alter the mode of microtubule sliding or bending. The role of the spoke and nexin components in movement is unknown, but the latter deserves some comment. Cilia and flagella differ with regard to the nexin linkages which maintain their 9-fold symmetry. Following low-ionic-strength fractionation, the 9 outer fibre doublets of *Tetrahymena* retain their cylindrical arrangement, whereas outer fibres of sea-urchin sperm (*Arbacia punctulata*) break down into lateral sheets of 9 and fewer (Gibbons, 1965). Stephens (1970*b*, 1971) has demonstrated by thermal fractionation of the B-subfibres that the nexin protein (mol. wt. 165000) interconnects the A-subfibres of the flagellar outer fibre doublets. Thus the cylindrical arrangement of fractionated ciliary axonemes from *Aequipecten* and *Tetrahymena* is maintained by nexin connexions between all 9 of the A-subfibres, whereas the fragmented appearance of *Aequipecten* and *Arbacia* flagellar outer fibres suggests that the attachment of nexin fibres is more labile in flagella. Whether nexin fibres are permanent or transitory cross-bridges is not yet known. If they are permanent connexions, then it is these structures which restrict the amount of sliding that can take place between adjacent outer fibre doublets. On the other hand, considering that the nexin fibres are approximately 30 nm long (estimated from cross-sections) and that the maximum amount of sliding between adjacent outer fibres is of the order of 100 nm (Satir, 1967), permanent nexin bridges would seem implausible.

The author is particularly grateful to Dr Raymond E. Stephens for guidance and encouragement throughout this investigation, and to Dr Andrew G. Szent-Györgyi for his constructive criticism and extensive use of laboratory facilities. This work was submitted to Brandeis University in partial fulfilment of the requirements of the Ph.D. degree and was supported by the following grants from the National Institutes of Health: Research grant GM 15500 awarded to Dr R. E. Stephens; Microbiology Training Grant GM 01586; Biomedical Science Institutional Grant FR 07044; and grant GM 265 issued to the Physiology course of the Marine Biological Laboratory, Woods Hole.

REFERENCES

AIELLO, E. & SLEIGH, M. A. (1972). The metachronal wave of lateral cilia of *Mytilus edulis*. *J. Cell Biol.* **54**, 493–506.

ALLEN, R. D. (1968). A reinvestigation of cross-sections of cilia. *J. Cell Biol.* **37**, 825–831.

BEHNKE, O. & FORER, A. (1967). Evidence for four classes of microtubules in individual cells. *J. Cell Sci.* **2**, 169–192.

BRENNER, S. & HORNE, R. W. (1959). A negative staining method for high resolution electron microscopy of viruses. *Biochim. biophys. Acta* **34**, 103–110.

BRYAN, J. & WILSON, L. (1971). Are cytoplasmic microtubules heteropolymers? *Proc. natn. Acad. Sci. U.S.A.* **68**, 1762–1766.

CASPAR, D. L. D., COHEN, C. & LONGLEY, W. (1969). Tropomyosin: crystal structure, polymorphism and molecular interactions. *J. molec. Biol.* **41**, 87–107.

FEIT, H., SLUSAREK, L. & SHELANSKI, M. L. (1971). Heterogeneity of tubulin subunits. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2028–2031.

FINE, E. R. (1971). Heterogeneity of tubulin. *Nature, New Biol.* **233**, 283.

GIBBONS, B. H., FRONK, E. & GIBBONS, I. R. (1970). Adenosine triphosphatase activity of sea urchin sperm. *J. Cell Biol.* **47**, 71A.

GIBBONS, B. H. & GIBBONS, I. R. (1972). Flagellar movement and adenosine triphosphatase activity in sea urchin sperm extracted with Triton X-100. *J. Cell Biol.* **54**, 75–97.

GIBBONS, I. R. (1961). The relationship between the fine structure and direction of beat in gill cilia of a lamellibranch mollusc. *J. biophys. biochem. Cytol.* **11**, 179–205.

GIBBONS, I. R. (1963). Studies on the protein components of cilia from *Tetrahymena pyriformis*. *Proc. natn. Acad. Sci. U.S.A.* **50**, 1002–1010.

GIBBONS, I. R. (1965). Chemical dissection of cilia. *Archs Biol., Liège* **76**, 317–352.

GIBBONS, I. R. (1966). Studies on the adenosine triphosphatase activity of 14 S and 30 S dynein from cilia of *Tetrahymena*. *J. biol. Chem.* **241**, 5590–5596.

GIBBONS, I. R. (1967). Proteins associated with movement in cilia. *7th Int. Congr. Biochem. Symp. Abstr.*, Symp. VI-4, 5, p. 333. Tokyo: Maruzen.

GIBBONS, I. R. (1968*a*). The structure and composition of cilia. *Symp. Soc. Cell Biol.* **6**, 99–113.

GIBBONS, I. R. (1968*b*). The biochemistry of motility. *A. Rev. Biochem.* **37**, 521–546.

GIBBONS, I. R. & FRONK, E. (1972). Some properties of bound and soluble dynein from sea urchin sperm flagella. *J. Cell Biol.* **54**, 365–381.

GIBBONS, I. R. & GRIMSTONE, A. V. (1960). On flagellar structure in certain flagellates. *J. biophys. biochem. Cytol.* **7**, 697–716.

GIBBONS, I. R. & ROWE, A. J. (1965). Dynein: A protein with adenosine triphosphatase activity from cilia. *Science, N.Y.* **149**, 424–426.

GRAY, J. (1955). The movement of sea-urchin spermatozoa. *J. exp. Biol.* **32**, 775–801.

HALL, C. E. (1960). Measurement of globular protein molecules by electron microscopy. *J. biophys. biochem. Cytol.* **7**, 613–618.

HOPKINS, J. M. (1970). Subsidiary components of the flagella of *Chlamydomonas reinhardii*. *J. Cell Sci.* **7**, 823–839.

HUXLEY, H. E. (1963). Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. *J. molec. Biol.* **7**, 281–308.

JACOBS, M., HOPKINS, J. M. & RANDALL, J. T. (1968). The flagellar proteins of a paralyzed mutant of *Chlamydomonas reinhardii*. *J. Cell Biol.* **39**, 66A.

JACOBS, M. & MCVITTIE, A. (1970). Identification of the flagellar proteins of *Chlamydomonas reinhardii*. *Expl Cell Res.* **63**, 53–61.

KIRKPATRICK, J. B., HYAMS, L., THOMAS, V. L. & HOWLEY, P. M. (1970). Purification of intact microtubules from brain. *J. Cell Biol.* **47**, 384–394.

LINCK, R. W. (1970). A biochemical comparison of ciliary and flagellar axonemes from the bay scallop, *Aequipecten irradians*. *Biol. Bull. mar. biol. Lab., Woods Hole* **139**, 429.

LINCK, R. W. (1973). Comparative isolation of cilia and flagella from the lamellibranch mollusc, *Aequipecten irradians*. *J. Cell Sci.* **12**, 345–367.

MAZIA, D., CHAFFEE, R. R. & IVERSON, R. M. (1961). Adenosine triphosphatase in the mitotic apparatus. *Proc. natn. Acad. Sci. U.S.A.* **47**, 788–790.

MOHRI, H. (1964). Extraction of ATPase from sea urchin and fish sperm tails. *Biol. Bull. mar. biol. Lab., Woods Hole* **127**, 381.

MOHRI, H., HASEGAWA, S., YAMAMOTO, M. & MURAKAMI, S. (1969). Flagellar adenosinetriphosphatase (dynein) from sea-urchin spermatozoa. *Scient. Pap. Coll. gen. Educ. Tokyo* **19**, 195–217.

OLMSTED, J. B., WITMAN, G. B., CARLSON, K. & ROSENBAUM, J. (1971). Comparison of the microtubule proteins of neuroblastoma cells, brain and *Chlamydomonas* flagella. *Proc. natn. Acad. Sci. U.S.A.* **68**, 2273–2277.

PHILLIPS, D. (1970). Insect sperm: Their structure and morphogenesis. *J. Cell Biol.* **44**, 243–277.

RAFF, E. C. & BLUM, J. J. (1969). The fractionation of glycerinated cilia by adenosine triphosphate. *J. biol. Chem.* **244**, 366–376.

RINGO, D. L. (1967). The arrangement of subunits in flagellar fibers. *J. Ultrastruct. Res.* **17**, 266–277.

SATIR, P. (1965). Studies on cilia. II. Examination of the distal region of the ciliary shaft and the role of the filaments in motility. *J. Cell Biol.* **26**, 805–834.

SATIR, P. (1967). Morphological aspects of ciliary motility. *J. gen. Physiol.* **50**, 241–258.

SCHACHMAN, H. K. (1959). *Ultracentrifugation in Biochemistry*. New York: Academic Press.

SHAPIRO, A. L., VIÑUELA, E. & MAIZEL, J. V. JR. (1967). Molecular weight estimation of polypetide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. biophys. Res. Commun.* **28**, 815–820.

SLEIGH, M. A. (1962). The biology of cilia and flagella. In *International Series of Monographs on Pure and Applied Biology*, vol. 12 (ed. G. A. Kerkut). New York and London: Pergamon Press.

STEPHENS, R. E. (1970*a*). Thermal fractionation of outer fiber doublet microtubules into A- and B-subfiber components: A- and B-tubulin. *J. molec. Biol.* **47**, 353–363.

STEPHENS, R. E. (1970*b*). Isolation of nexin – the linkage protein responsible for the maintenance of the nine-fold configuration of flagellar axonemes. *Biol. Bull. mar. biol. Lab., Woods Hole* **139**, 438.

STEPHENS, R. E. (1971). Microtubules. In *Biological Macromolecules*, vol. 5 (ed. S. N. Timasheff & G. D. Fasman), pp. 355–391. New York: Marcel Dekker.

STEPHENS, R. E. (1972). Studies on the development of the sea urchin *Strongylocentrotus droebachiensis*. III. Embryonic synthesis of ciliary proteins. *Biol. Bull. mar. Biol. Lab., Woods Hole* **142**, 489–504.

SUMMERS, K. E. & GIBBONS, I. R. (1971). Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea urchin sperm. *Proc. natn. Acad. Sci. U.S.A.* **68**, 3092–3096.

SZENT-GYÖRGYI, A. G. (1953). Meromyosins, the subunits of myosin. *Archs Biochem. Biophys.* **42**, 305–320.

WARNER, F. D. (1970). New observations on flagellar fine structure. The relationship between matrix structure and the microtubule component of the axoneme. *J. Cell Biol.* **47**, 159–182.

WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. biol. Chem.* **244**, 4406–4412.

WEISENBERG, R. C. & TAYLOR, E. W. (1968). Studies on ATPase activity of sea urchin eggs and the isolated mitotic apparatus. *Expl Cell Res.* **53**, 372–384.

WITMAN, G. B. (1970). Fractionation and biochemical characterization of the flagella of *Chlamydomonas reinhardii*. *J. Cell Biol.* **47**, 229A.

(*Received 7 September* 1972)

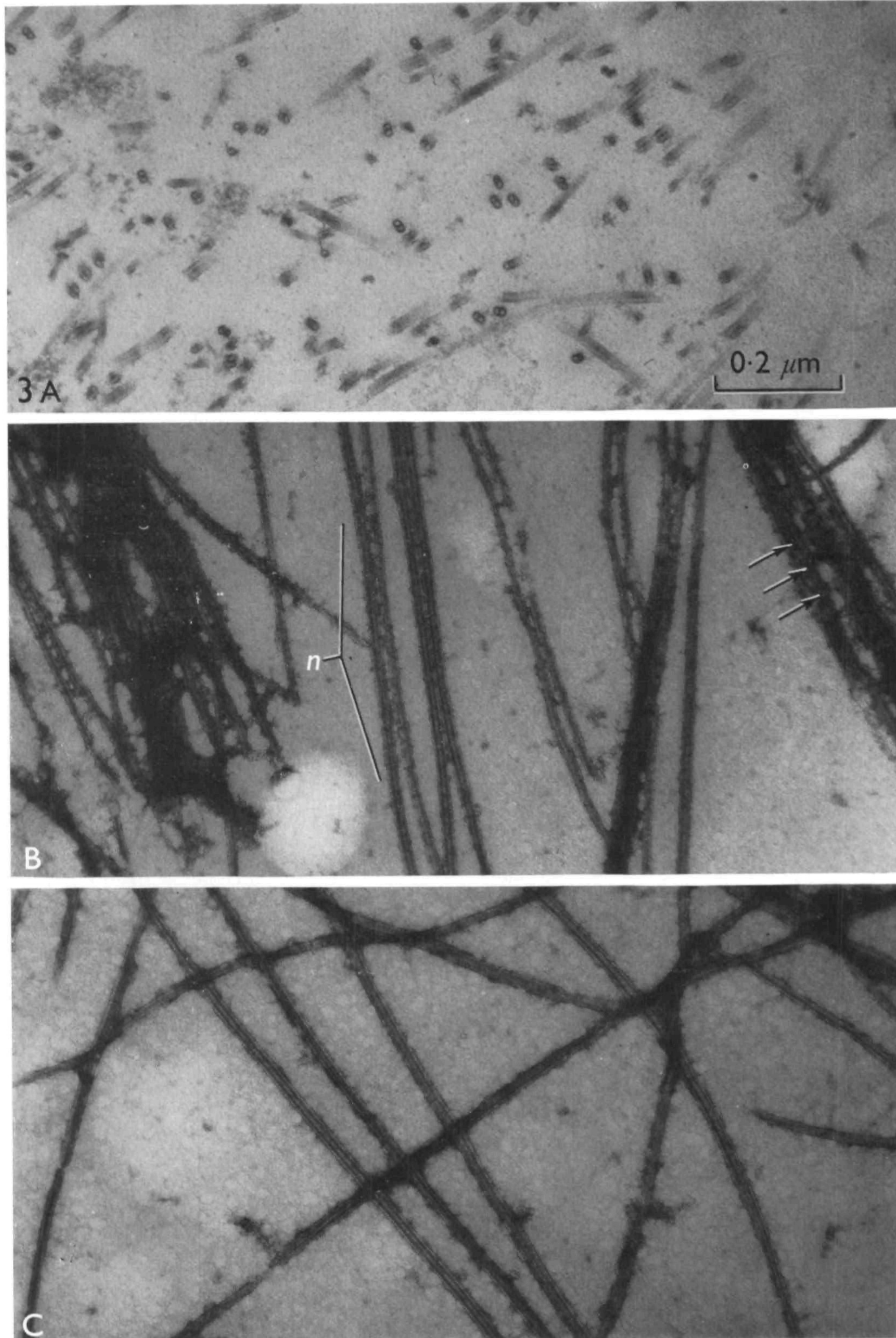


Fig. 3. For legend see p. 970.

Fig. 3. The pellet fraction from a Tris-EDTA dialysis of *A. irradians* flagellar axonemes. A, the arms, spoke material and nexin fibres have been solubilized, leaving only unlinked, doublet outer fibres; $KMnO_4$/lead citrate. B, C, preparations of flagellar outer fibres negatively stained following a 24-h (B) and a 48-h (C) dialysis. Arrows indicate the few nexin linkage fibres remaining after an initial 24-h dialysis. The bracket (*n*) spans a region of nexin bridges which appear to be frayed in a direction perpendicular to the microtubule axis. All 3 micrographs are at the same magnification.

Fig. 4. Pellet fraction from a 48-h Tris-EDTA dialysis of *A. irradians* ciliary axonemes. Fractionated axonemes are printed in what is believed to be their clockwise enantiomorphic form. In most cases the B-subfibre (*bt*) depolymerizes, leaving a dense granule (*g*) on or inside the A-subfibre; only A-subfibres, an amorphous matrix material and occasionally one central fibre remain. Arms are not observed on the A-subfibres, but radial links (*rl*) are occasionally seen as in G, diagrammed in H. The diagram H also illustrates the breakdown of the B-subfibres. Stain: A, D and G, uranyl acetate/lead citrate; B, C, E, and F, $KMnO_4$/lead citrate.

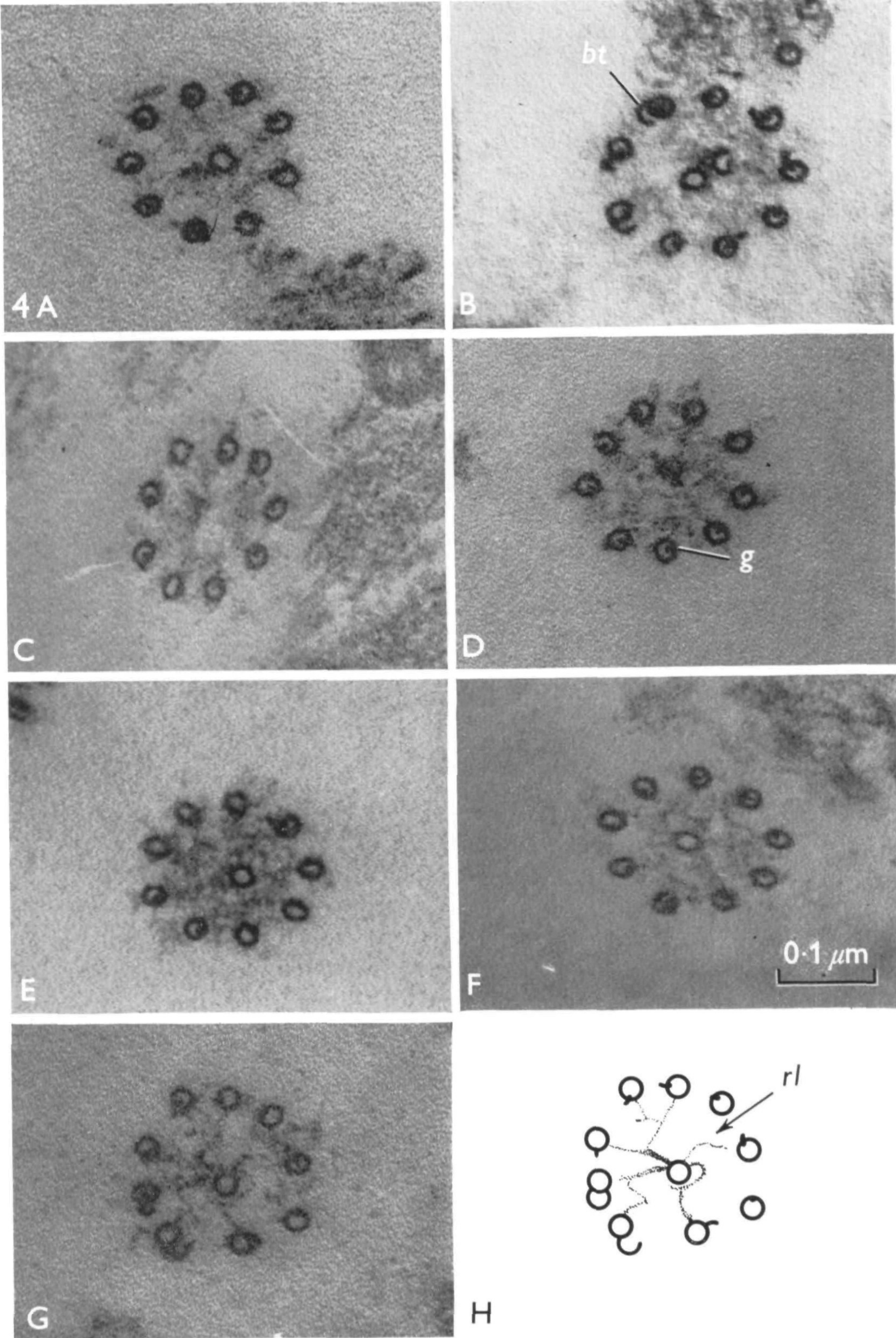
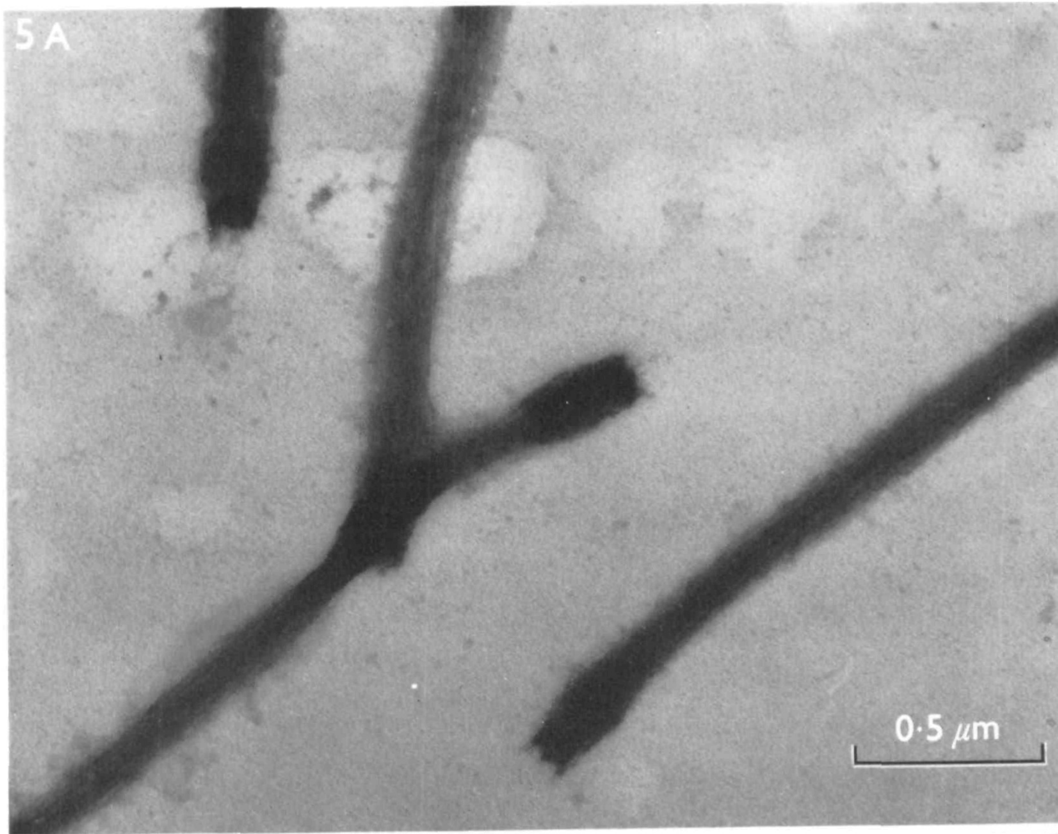
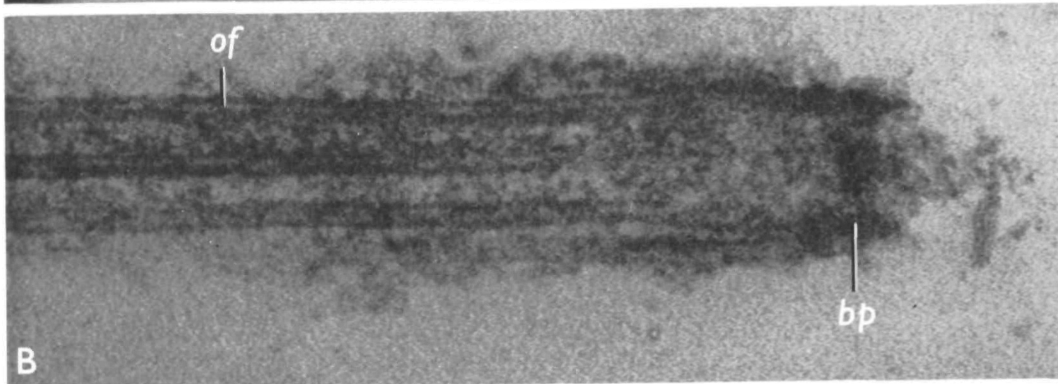

4 A
B
bt
C
D
g
E
F
0·1 μm
G
H
rl

Fig. 5. Preparations of ciliary singlet axonemes showing the transition zone. A, negatively stained with uranyl acetate; B, tangential section showing 3 A-subfibres (*of*) passing into the amorphous region of the transition zone. The ends of the broken fibres extend slightly past the basal plate (*bp*). C, medial section through the transition zone. The outer fibres maintain their double nature only within the transition region. Distal to this region the central fibre is missing but the matrix is filled with disoriented spoke material. B and C are at the same magnification; both are stained with $KMnO_4$/lead citrate.


5 A
0·5 μm


of
bp
B

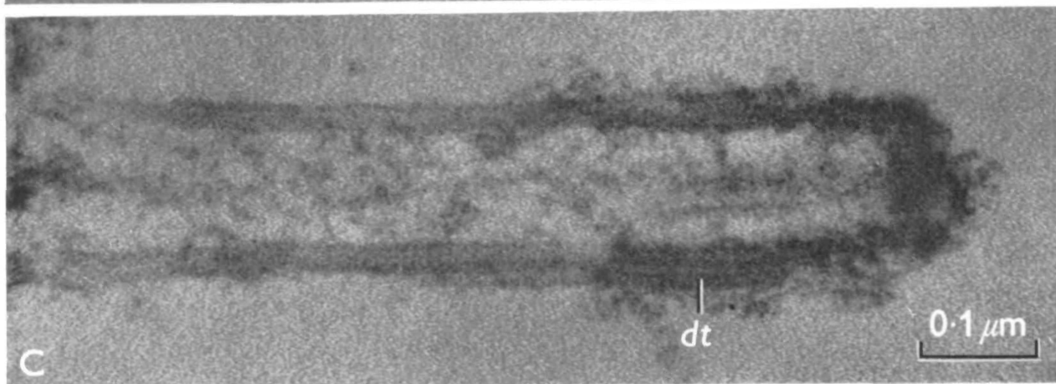
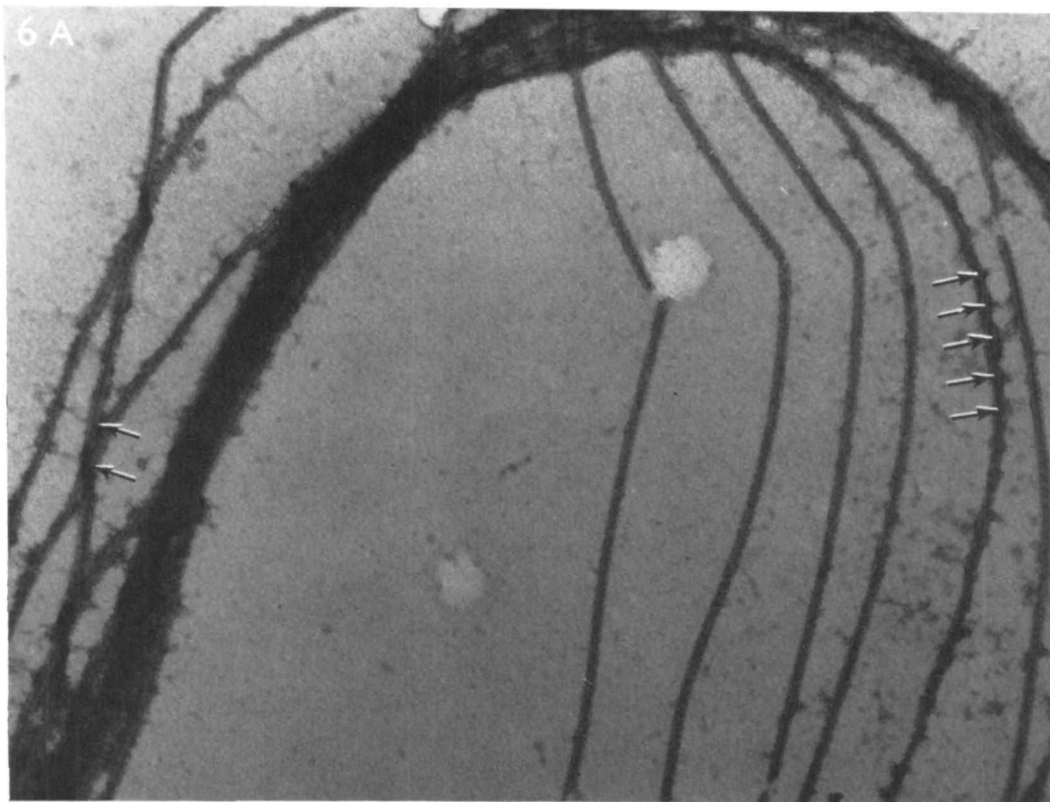

dt
0·1 μm
C

Fig. 6. Negatively stained preparations of frayed ciliary singlet axonemes. In A, nexin linkage fibres remain tightly applied to the A-subfibres (designated by arrows on right) and sometimes appear as thin filaments (arrows on left). In B, frayed A-subfibres are held firmly together at the basal end by the transition zone and basal plate. Stained with uranyl acetate. Both micrographs at same magnification.


6 A

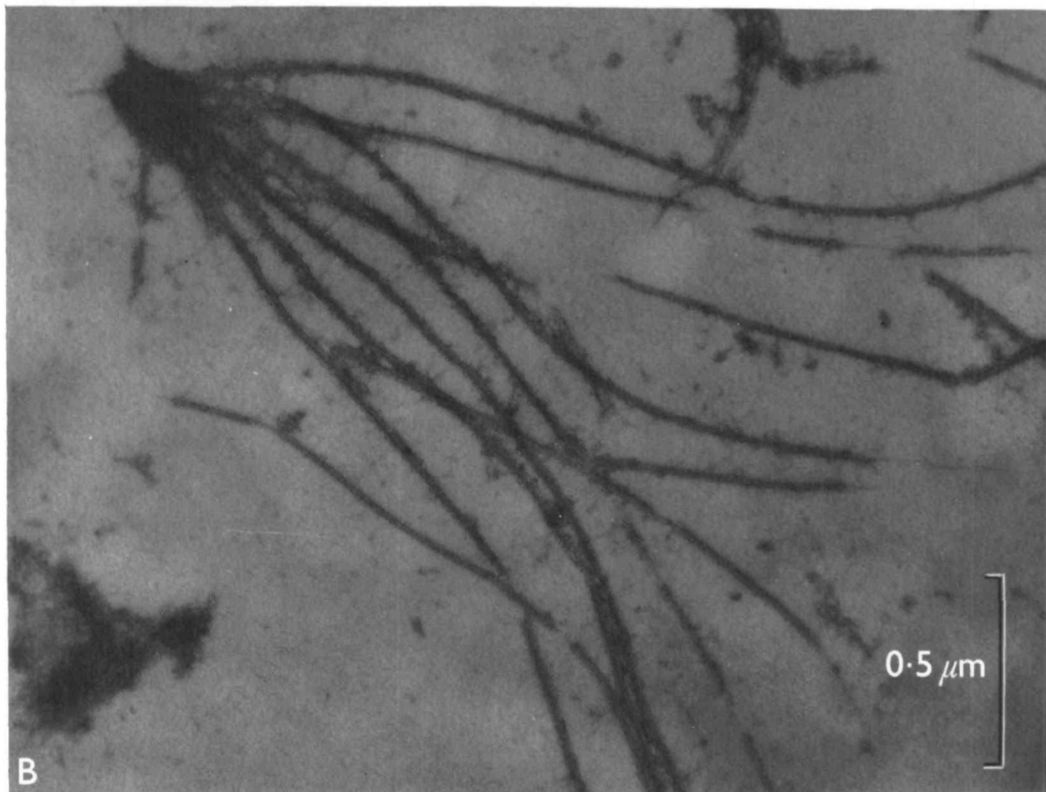

B
0·5 μm

Fig. 7. Analytical ultracentrifuge sedimentation velocity patterns. A, B, supernatant fractions from dialysed ciliary (A) and flagellar (B) axonemes of *A. irradians*. The ciliary fraction contains 3·5 and 10 S components; protein concentration, 9 mg/ml; temperature, 20·4 °C. Flagellar components measure 3·5 and 11 S; protein concentration, 5 mg/ml; temperature, 20·0 °C. The sedimentation coefficients were determined in 10 mM Tris, 0·1 mM EDTA, pH 8·0, at a speed of 59780 rev/min. Frames shown were taken 24 min after reaching speed; bar angle, 60°. C, ciliary pellet fraction from dialysis ('singlet axonemes') following a 2-min trypsin treatment and a subsequent 12-h dialysis against Tris-EDTA. Frame taken 40 min after reaching speed (59780 rev/min) shows 3 and 9 S components in 1 mM Tris, 0·1 mM EDTA, 0·1 % mercaptoethanol, pH 8·0; protein concentration, 7 mg/ml; temperature, 1·1 °C; bar angle, 60°.

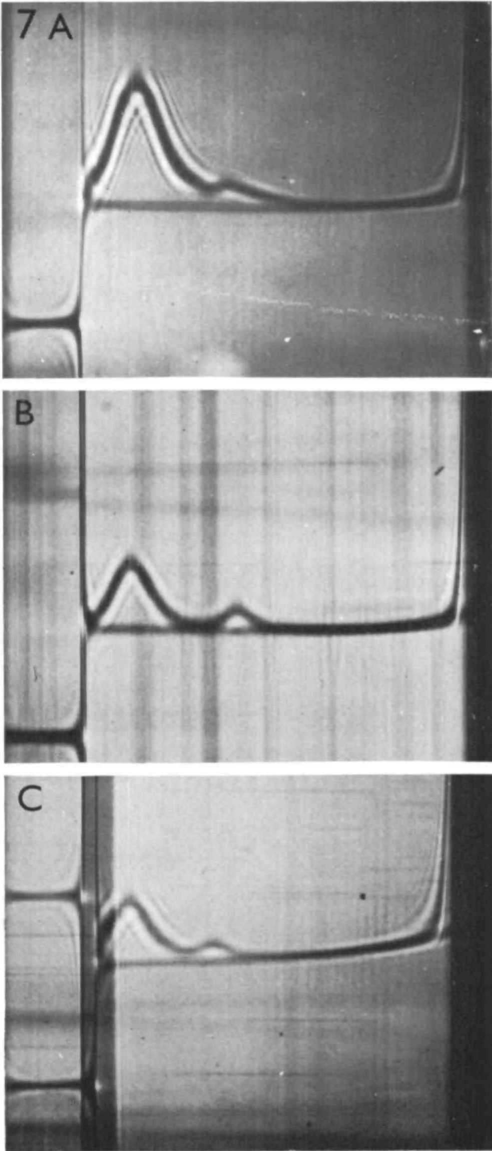
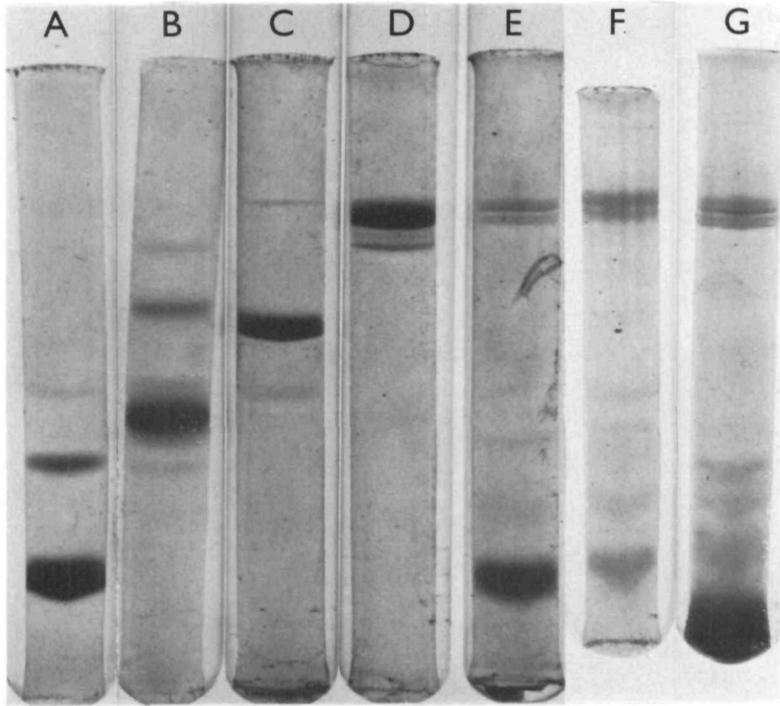

7 A
B
C

Fig. 8. SDS-polyacrylamide gels (3 %) of 14 S ciliary dynein and mol. wt. standards. Samples were applied in a medium containing 1 % SDS, 10 mM phosphate, pH 7·0, 10 % glycerol and 0·01 % bromophenol blue indicator. Reducing agent was added only where indicated. In all cases the sample volume was 25 μl. A, bovine serum albumin, 25 μg; B, gamma globulin, 25 μg; C, rabbit myosin in 0·1 % mercaptoethanol, 12 μg; D, rabbit myosin, 12 μg; E, ciliary supernatant fraction from Tris-EDTA dialysis, in 0·1 % mercaptoethanol, 25 μg; F, 14 S ciliary dynein partially purified by Sephadex G-200 chromatography of the dialysis supernatant fraction (specific activity enhanced from 0·31 to 1·21 μmol P_i/min/mg protein), 15 μg; G, ciliary dialysis supernatant fraction (unpurified) exposed to 8 M urea in SDS medium containing 0·1 % mercaptoethanol, 50 μg. Gels of bovine serum albumin and of gamma globulin show monomer, dimer and trimer bands in the absence of reducing agent. Gels of ciliary dialysis fractions show prominent bands at approximately 60000 mol. wt. (tubulin) and at 450000 and 500000 (the 2 components of 14 S dynein). The occasional doubleness of the 2 dynein components is believed to be an artifact of the gel system. Gels A–E are of the same set and were used to plot Fig. 2.

Fig. 9. Results of the subfraction of the ciliary pellet fraction (singlet axonemes) on SDS-acrylamide gels (3 %). A, ciliary pellet fraction untreated, 82 μg in 50 μl; B, after 2 min trypsin treatment at 20 °C, 80 μg in 50 μl; C, same as B but after a 6-h dialysis against Tris-EDTA, 75 μg in 50 μl; D, supernatant obtained after extraction of the singlet axonemes with 2 % sarkosyl, 10 mM Tris, pH 8·0, 0·1 mM EDTA, and 0·1 mM dithiothreitol, at room temperature for 30 min and centrifuging for 20 min at 100000 *g*, 66 μg in 40 μl; E, twice-extracted pellet from D, 18 μg in 25 μl and overloaded with respect to its percentage of the whole singlet axoneme fraction; F, singlet axoneme fraction in 8 M urea plus SDS medium, 50 μg in 25 μl.


8
A
B
C
D
E
F
G
14 S dynein
components:
500000 mol. wt.
450000 mol. wt.

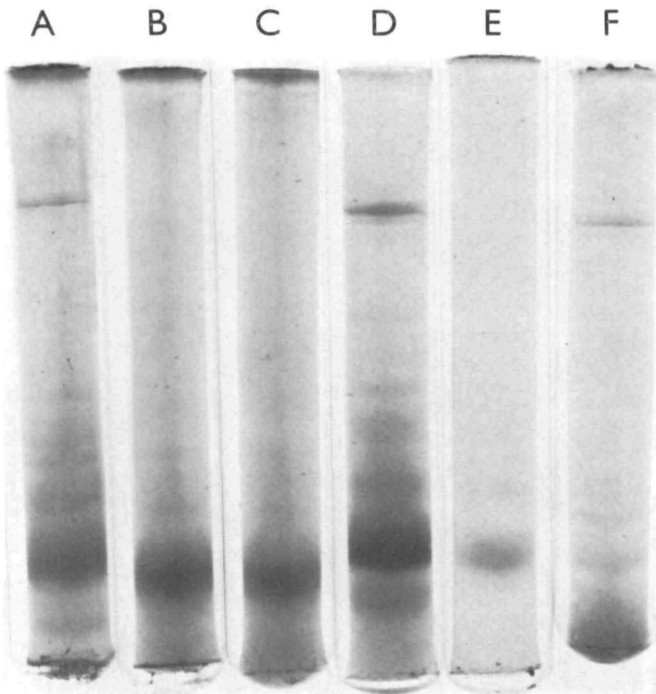

A
B
C
D
E
F
9
Tightly bound
dynein 500000
mol. wt.

Fig. 10. SDS-polyacrylamide gels (3 %) of whole and fractionated ciliary and flagellar axonemes. Gels within a group were polymerized from the same mixture and run simultaneously.

A–C. A, ciliary axonemes from cilia isolated with hypertonic seawater; B, ciliary axonemes from cilia isolated with ethanol/calcium; C, flagellar axonemes from sperm. All from *Aequipecten irradians*. Loading concentration, 84 μg of protein in 25 μl of SDS-medium. *d*, 14 S material; *t*, 60000 mol. wt. tubulin monomer.

D, E. D, ciliary axonemes from *A. irradians*; E, flagellar axonemes from *Strongylocentrotus droebachiensis* (sea urchin) sperm. Loading concentration, 57 μg in 25 μl.

F, G. F, flagellar axonemes from *A. irradians*; G, flagellar axonemes from *S. droebachiensis*. Loading concentration, 25 μg in 25 μl.

H–J. Fractionation of ciliary axonemes from *A. irradians*. H, whole, unfractionated axonemes, 57 μg in 25 μl; I, supernatant fraction, 16 μg in 25 μl; J, pellet fraction, 41 μg in 25 μl. The supernatant and pellet fractions have been loaded stoichiometrically with respect to the gel of whole axonemes.

K, L. Fractionation of flagellar axonemes from *A. irradians*. K, whole, unfractionated axonemes, 85 μg in 25 μl; L, pellet fraction, 78 μg in 50 μl. The pellet fraction has been overloaded with respect to its true percentage of the whole axoneme. The lower dynein band in K is somewhat diffuse and thus does not show up well in the photograph.

M–O. Fractionation of flagellar axonemes from *S. droebachiensis*. M, whole, unfractionated axonemes, 100 μg in 50 μl; N, supernatant fraction, 33 μg in 50 μl; O, pellet fraction, 67 μg in 50 μl. Supernatant and pellet fractions were loaded stoichiometrically with respect to the gel of whole axonemes. To each gel, 25 μg of haemoglobin has been added as a marker, appearing as the broad band at the bottom in gels M–O. The dynein band remaining in the pellet fraction (O) appears to migrate with the lower band of the supernatant and whole axonemes, but in fact its mobility (R_F) corresponds to that of the upper, 500000-Dalton protein.

10