COMPARATIVE ISOLATION OF CILIA AND FLAGELLA FROM THE LAMELLIBRANCH MOLLUSC, AQUIPECTEN IRRADIANS

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SUMMARY

Gill cilia and sperm flagella from the lamellibranch mollusc Aequipecten irradians were compared with respect to their ultrastructures and adenosinetriphosphatase activities. Cilia were isolated from excised gills using 3 different solutions: twice-concentrated seawater, 10% ethanol-10 mM CaCl₂, and 60% glycerol. In each case deciliation occurs by the severance of the cilium at the junction of the transition zone and the basal body, and in each case the ciliary ultrastructure is maintained. Sperm flagella were purified by mechanical decapitation. Cilia and sperm flagella have similar fine structures, except that the matrix of the cilia contains substantially more electron-dense material than that of flagella. The ATPase activity of purified cilia is approximately 0.09 μmol P₄/min/mg protein; that of flagella is 0.13. Ciliary and flagellar axonemes were prepared by repeated extraction of the membranes with 1% Triton X-100. Ciliary axonemes maintain their 9 + 2 cylindrical orientation, whereas flagellar axonemes often appear as opened or fragmented arrays of the 9 + 2 structure, due to the partial breakdown of the flagellar nexin fibres. A-subfibre arms which were obvious in whole organelles are rarely seen in axoneme preparations. Again the ciliary matrix is considerably more amorphous than in flagellar axonemes. The ATPase activities of ciliary and flagellar axonemes are 0.13 and 0.12 μmol P₄/min/mg protein respectively; however, activities of ciliary axonemes may vary by a factor of 2, depending on the method of isolation. The difficulty in observing A-subfibre arms in cross-sections of ciliary and flagellar axonemes is discussed in terms of random, non-reinforcing arrangements of the dynein arms.

INTRODUCTION

A number of investigators have reported methods for the isolation of cilia and flagella and have characterized the major components of these organelles (Gibbons, 1968; Stephens, 1971), but a comparison of cilia and flagella from the same species has not been reported. Considering that cilia and flagella differ in their modes of beat (Gray, 1928, 1955; Sleigh, 1962), such intraspecies comparisons might reveal structural or biochemical differences relevant to the phenomenon of wave propagation.

Since lamellibranch molluscs provide a constant source of gill cilia and an adequate, seasonal supply of sperm (Sastry, 1970), the bay scallop Aequipecten irradians was investigated. Three previous methods of cilia isolation were modified for harvesting gill cilia (Auclair & Siegel, 1966; Gibbons, 1965a, b; Watson & Hopkins, 1962). The 3 isolation methods were compared on the basis of the yields, the ATPase activities

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and the ultrastructure of whole cilia and Triton-extracted ciliary axonemes. Flagella and flagellar axonemes were obtained from ripe scallops and similarly investigated. This report deals with the physiological and structural differences between the isolated organelles. Ciliary and flagellar axonemes from *A. irradians* were then chemically fractionated and found to differ in their forms of dynein and in the stabilities of their homologous microtubules and secondary structures; these results are presented in the following paper (Linck, 1973).

**MATERIALS AND METHODS**

**Solutions, reagents and conditions**

Solutions frequently used in this report have been abbreviated as follows: *Tris-Mg solution* denotes 30 mM Tris (tris(hydroxymethyl)aminomethane), 3 mM MgCl₂, 0.1 mM EDTA (ethylenediaminetetra-acetate), pH 8.3 at 0 °C; *Tris-Mg-Triton solution*, 30 mM Tris, 3 mM MgCl₂, 0.1 mM EDTA, 1% Triton X-100, pH 8.3, at 0 °C; and *EDTA-seawater*, filtered seawater made 0.1 mM in EDTA. Adenosine triphosphate, obtained from Calbiochem and P-L Biochemicals, was stored as the powder or as a neutralized 0.1 M stock solution in a −20 °C deep freeze. All procedures were carried out at 0 °C unless otherwise specified. The cheesecloth used in the cilia and sperm tail isolation procedures was first soaked in EDTA-seawater.

**Procedures for the isolation of gill cilia**

Lamellibranch molluscs of the species *Aequipecten irradians* (formerly *Pecten irradians*) were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. The gills were excised, collected in roughly 10 times their volume of cold *EDTA-seawater* and washed twice more by transfer to fresh *EDTA-seawater* to remove silt and mucous. In some cases the wet weight of the gills was recorded in order to calculate yields. Three previous methods of cilia isolation were modified for harvesting gill cilia:

1. **The 2 × seawater procedure** (Auclair & Siegel, 1966; Stephens & Linck, 1969). Gills were suspended in roughly 10 times their volume of twice-concentrated *EDTA-seawater* (30 g of NaCl per l. of *EDTA-seawater*) at room temperature and stirred gently but continuously for 10 min, at which point a maximally cloudy suspension of cilia and fragmented gills was obtained. It was noted that in this procedure the gills do not deciliate as readily in cold hypertonic seawater. The suspension was filtered through cheesecloth and then centrifuged at 1500 g for 5 min in 50-ml tubes or for 10 min in 250- or 300-ml bottles. The supernatants were carefully collected and recentrifuged at 10000 g for the same length of time to pellet the essentially pure cilia.

2. **The ethanol—calcium procedure** (Watson & Hopkins, 1962; Gibbons, 1965a). Each 10 g of gills was suspended in 100 ml of medium consisting of 10% ethanol, 0.1 M NaCl, 2 mM EDTA, and 30 mM Tris, pH 8.0, at room temperature. The gills were stirred and allowed to equilibrate for 2 min, after which the suspension was made 10 mM in CaCl₂ by rapidly adding from a 1 M stock solution and stirring continuously. At room temperature the deciliation began immediately and reached a maximum in about 4 min (about twice as long at 0 °C). The suspension was filtered through cheesecloth and then centrifuged at 1500 g for 5 min in 50-ml tubes or for 10 min in 250- or 300-ml bottles. The supernatants were carefully collected and recentrifuged at 10000 g for the same length of time to pellet the essentially pure cilia.

3. **The glycerol procedure** (Gibbons, 1965b). Approximately 20 g of wet gills were suspended in 200 ml of 70% glycerol, 30 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, and 30 mM Tris, pH 8.0, at room temperature, and stirred continuously for 15 min. After filtering through a thin layer of cheesecloth, the suspension was cooled to 0 °C and centrifuged at 12000 g for 10 min using half-full 50-ml tubes. The supernatant was carefully collected and centrifuged at 80000 g for 3 h to pellet the cilia.

The pellets of cilia obtained from a given procedure were consolidated and washed once with 20 vol. of *Tris-Mg solution* for use as whole cilia, or washed 3 times with 20 vol. of *Tris-Mg-Triton solution*, followed by 1 wash with *Tris-Mg solution*, for use as ciliary axonemes. Three washes of the *Tris-Mg-Triton solution* were found to remove essentially all of the ciliary
membranes. The washed cilia or ciliary axonemes were collected by centrifugation at 10,000 g for 5 and 10 min, respectively. Cilia preparations were routinely examined under a phase-contrast microscope for purity.

**Procedure for sperm tail isolation**

The gonads were excised from ripe scallops and the testes were trimmed free of the adjacent ovaries using stainless steel forceps and scissors. The testes were collected in a beaker on ice, cut into quarters, and the sperm extracted with 5 vol. of ice-cold EDTA-seawater for 30 min with stirring. The extruded sperm were decanted through cheese cloth, and the minced testes were re-extracted twice more. The exudates were centrifuged at 5,000 g for 5 min to pellet the sperm. (Cleaner, whiter sperm pellets were obtained if the gonads were first wiped clean of their black pigment with a tissue.) After resuspension in 20 vol. of EDTA-seawater, the sperm were checked in a phase-contrast microscope for motility and purity and then decapitated by homogenizing for 2 s at full speed in a Sorvall Omni-Mixer, following the procedure used by Stephens (1970a). The preparation was again observed for completeness of decapitation and then filtered through cheese cloth to remove the layer of foam. Sperm heads were removed by centrifugation at 10,000 g for 5 min, and the opalescent supernatant of tails was carefully decanted. If by microscopic examination the pellet contained an appreciable number of tails, it was re-suspended and again centrifuged. The supernatants were then centrifuged at 10,000 g for 5 min to sediment the tails. The purified tails were then washed with Tris-Mg solution or extracted twice with Tris–Mg–Triton solution followed by a Tris–Mg wash to yield pellets of flagella and flagellar axonemes, respectively. Flagellar axonemes were sedimented by centrifugation for 10 min at 10,000 g.

**Protein determination**

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as a standard.

**Adenosinetriphosphatase activity**

ATPase activity was assayed according to the procedure used by Stephens & Levine (1970). The ATPase assay medium, containing 1 mM ATP (neutralized to pH 7 with NaOH), 30 mM Tris buffer (pH 8.0 at 20 °C), 1 mM MgCl₂ and 0.1 mM EDTA, was usually made up in quantity (500 ml) and divided using an automatic pipette into 5-ml aliquots which were kept frozen at −20 °C until use. Prior to assay the required number of aliquots were thawed for 5–10 min in a waterbath at 20 °C. Immediately after starting the reaction by the addition of a known volume of enzyme (not exceeding 0.5 ml), a 2.0-ml sample was withdrawn and rapidly mixed with 0.2 ml of 50 % trichloroacetic acid in an ice bath. This sample represented the zero time blank. A second 2.0-ml sample was withdrawn at 10 min. The TCA-precipitated protein was removed by spinning for 3 min at top speed in a clinical centrifuge, and the supernatants were assayed for inorganic phosphate by the method of Taussky & Schorr (1952). A 1.8-ml aliquot of supernatant was mixed with 0.5 ml of 2 % ammonium molybdate in 2 N sulphuric acid, followed by the addition of 0.2 ml of freshly prepared ferrous sulphate solution (2 g in 5 ml of 1 N sulphuric acid). The optical densities of the 10-min samples were read immediately against their respective zero time blanks; duplicate assays agreed to within ±5 %. A value of 0.833 μmol of phosphate (per 2.5 ml assay) per optical density unit was determined from a standard phosphate curve, and the specific ATPase activity was based on the μmol of phosphate liberated per min per mg protein.

**Electron microscopy**

Materials for electron microscopy were fixed for 1 h in either 1 % osmium tetroxide buffered at pH 7.4 with veronal acetate (Pease, 1964) or in 3 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Following glutaraldehyde fixation, the samples were washed for three 20–30 min periods in 0.1 M phosphate buffer, pH 7.4, and then postfixed for 1 h with osmium tetroxide as above. Osmium-fixed and osmium-postfixed samples were dehydrated by a series of 20–30 min
washes in 70, 85, 90 and 95% ethanol and 2 washes in 100% ethanol and finally by two 15-min washes in propylene oxide. For embedding a 1:1 mixture (v/v) of Araldite 506 and dodecenyl succinic anhydride (DDSA) and a final concentration of 1.5% catalyst (dimethylaminomethyl-phenol or DMP-30) was prepared and kept frozen until use. The samples were treated for 1 h in a 1:1 solution (v/v) of propylene oxide and the Araldite-catalyst mixture and then for 3-6 h in a 1:3 solution of the same. The samples were finally embedded in the pure Araldite-catalyst mixture and hardened for 3-4 days at 60°C. Sectioning was performed with a Reichert Om U2 ultramicrotome using a diamond knife. Most sections used in this study were approximately 60 nm thick, as judged from their silver interference colours after flattening with chloroform vapour. The sections were picked up using parlodion-coated 400-mesh grids, and subsequently stained by one of 2 procedures:

1. In most cases the sections were stained for 30 min with saturated uranyl acetate in 50% methanol (protected from light), rinsed with distilled water and dried. They were then stained for 15 min with Reynolds's (1963) lead citrate, rinsed with 0.01% NaOH and then with water and allowed to dry.

2. Occasionally sections were stained with 1% potassium permanganate for 1-5 min, rinsed with 5% citric acid and then with water, allowed to dry and stained with Reynolds's lead citrate. Photographs were taken on a Philips 300 electron microscope fitted with a 30-nm objective aperture and operated at 60 kV. Calibration of the magnification was based on the 39.5-40.0 nm spacing of Mg2+-taetoids of rabbit tropomyosin (provided by Dr Andrew G. Szent-Györgyi).

RESULTS

Comparative yields and enzymic activities of isolated cilia

In the series of experiments shown in Table 1, the hypertonic seawater, the ethanol-calcium and the glycerol treatments produce comparable yields of cilia with similar specific ATPase activities. A typical batch of 25 scallops (6.4-7.6 cm in diameter) contains about 50 g of gills (wet weight) and yields about 50 mg of whole cilia with a specific, Mg2+-activated ATPase activity of 0.09 µmol of P_i/min/mg protein. Compared to the relatively brief 2 x seawater and ethanol-calcium procedures, the glycerol method is time consuming and technically more difficult, requiring ultracentrifugation of large volumes of cold 60% glycerol.

In order to remove the ciliary membrane and soluble matrix material, the isolated cilia were extracted 3 times with the detergent Triton X-100. As shown in Table 1, the specific ATPase activity of the resulting axonemes is approximately 0.13 µmol P_i/min/mg protein, and in these initial 5 studies this value appeared to be independent of the method of cilia isolation.

Cilia isolated by the 3 procedures differ in their amounts of membrane and matrix protein. Extraction with Triton removes approximately 56% of the protein from glycerol-isolated cilia but only 30 or 38% from cilia isolated by 2 x seawater or ethanol-calcium respectively. Thus, the glycerol treatment may release a significant amount (20%) of contaminating, non-ciliary membranes, or conversely, it may preserve a greater amount of ciliary matrix and/or membrane material than the other methods of isolation. Regardless, these values should be compared with the findings of Gibbons (1965a) that the digitonin-extracted membrane and matrix components of Tetrahymena cilia account for approximately 50% of the ciliary protein.

The enzymic properties of Aequipecten ciliary axonemes have been previously studied (Stephens & Levine, 1970), and, in particular, the specific ATPase activity was found
Cilia and flagella from a mollusc

Table 1. Comparative yields and ATPase activities of isolated cilia and ciliary axonemes

<table>
<thead>
<tr>
<th></th>
<th>2 x seawater</th>
<th>Ethanol/calcium</th>
<th>60 % glycerol</th>
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<tr>
<td>Ciliary protein, mg/g of gill</td>
<td>0.32</td>
<td>0.92</td>
<td>0.45</td>
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<td></td>
<td>0.99</td>
<td>0.97</td>
<td>1.26</td>
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<td></td>
<td>1.01</td>
<td>1.25</td>
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<tr>
<td></td>
<td>0.02</td>
<td>1.55</td>
<td></td>
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<tr>
<td>Specific ATPase activity of cilia</td>
<td>0.05</td>
<td>0.09</td>
<td>0.07</td>
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<tr>
<td></td>
<td>0.09</td>
<td>0.10</td>
<td>0.08</td>
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<tr>
<td></td>
<td>0.09</td>
<td>0.12</td>
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<tr>
<td></td>
<td>0.10</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Axonemal protein, mg/g of gill</td>
<td>0.38</td>
<td>0.63</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>0.38</td>
<td>0.63</td>
<td>0.27</td>
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<tr>
<td></td>
<td>0.60</td>
<td>0.63</td>
<td>0.67</td>
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<td></td>
<td>0.73</td>
<td>0.69</td>
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<td></td>
<td>0.85</td>
<td>1.10</td>
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<tr>
<td>Specific ATPase activity of axonemes</td>
<td>0.11</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>0.13</td>
<td>0.12</td>
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<tr>
<td></td>
<td>0.14</td>
<td>0.13</td>
<td>0.14</td>
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<tr>
<td></td>
<td>0.15</td>
<td>0.14</td>
<td></td>
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<tr>
<td>Protein remaining after Triton, %</td>
<td>57</td>
<td>69</td>
<td>36</td>
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<tr>
<td></td>
<td>58</td>
<td>58</td>
<td>53</td>
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<tr>
<td></td>
<td>70</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>71</td>
<td></td>
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<tr>
<td>Enzyme units remaining after Triton, %</td>
<td>80</td>
<td>106</td>
<td>54</td>
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<tr>
<td></td>
<td>82</td>
<td>76</td>
<td>77</td>
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<td></td>
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<td>136</td>
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Specific ATPase activity is given in terms of $\mu$mol $P_i$/min/mg protein, enzyme units as $\mu$mol $P_i$/min; values in columns are from different experiments and those in bold type are the averages.

to be $0.11 \pm 0.01 \mu$mol $P_i$/min/mg protein. Certain other characteristics concerning the specific ATPase activity of the axoneme fraction have been noted in the present study. In a series of 15 isolations of cilia prepared from hypertonic seawater, the specific ATPase activities of the axonemes ranged from 0.12 to 0.24 with an average and standard deviation of 0.17 $\pm$ 0.04 $\mu$mol $P_i$/min/mg protein, a value which is 30% higher than that obtained from the initial 5 studies of Table 1. The specific ATPase activities of axonemes stored on ice as a suspension in Tris-Mg solution were also observed to fluctuate with the age of the preparation. Generally the activity increased about 25% during the first day after isolation and decreased about 10% each day thereafter. It was further noted, somewhat unexpectedly, that the amount of original ciliary enzyme units remaining after Triton extraction varied according to the method.
Axonemes from glycerol-isolated cilia contain approximately 66% of the total ciliary enzyme units, in accordance with the findings of Gibbons (1965a) for *Tetrahymena* cilia that 70% of the enzymic activity is associated with the axoneme, while the remaining 30% is membrane bound. The ATPase activity of axonemes isolated from hypertonic seawater and ethanol-calcium appears to be increased following extraction with Triton, so that in these cases no accurate estimates can be made of the relative amounts of axoneme and membrane ATPases.

**Ultrastructure of intact and isolated cilia and of ciliary axonemes**

Pellets of whole cilia from each of the 3 isolation procedures, axonemes of cilia isolated by 2x seawater, and a piece of fresh gill were fixed for electron microscopy; samples of the optimally fixed materials are illustrated in Figs. 1-4. The gill fragment and whole cilia preparations were sufficiently fixed by osmium tetroxide alone, whereas the axonemes seemed to require fixation with glutaraldehyde and a postfixation with osmium tetroxide. The purest cilia preparations were obtained using the hypertonic seawater method of isolation (Fig. 1); the ethanol–calcium and the glycerol procedures were observed to fragment the gill to a greater degree during the isolation, resulting in some contamination by disrupted epithelial membranes and cytoplasmic debris.

Essentially all of the fine structure observed in gill cilia *in situ* (Fig. 2) is maintained in the isolated organelles (Fig. 3). Cilia isolated from 2x seawater and from ethanol–calcium are essentially indistinguishable (Fig. 3A–H); glycerol-isolated cilia (Fig. 3I–L) are unique in that considerably more amorphous material coats the outer fibre doublets and fills the matrix region. Following isolation the outer fibre doublets retain their cylindrical 9 + 2 relationship (approximately 190 nm in diameter) and their skewed orientation of approximately 10 degrees (i.e. the angle subtended by the axial plane of the doublet and the tangent to the axoneme at the centre of the fibre doublet). Infrequently one or two nexin fibres (cf. Stephens, 1970b, 1971) can be seen in cross-section linking adjacent doublet outer fibres, but it is not possible here to ascertain the A- to A-subfibre connexions. The paired arms of cilia *in situ* are sharply delineated asymmetric structures attached to the A-subfibres. The outer arm is sharply hooked and bent inwards to varying degrees, whereas the inner member of the pair may curve either inwards or outwards. Thus the pair of arms may assume either a ‘lobster claw’ appearance or the asymmetric pattern described for *Tetrahymena* by Allen (1968). In general, the frequency and asymmetry of the arms are preserved in the isolated cilia, although the arms are usually much less densely stained. Arms are rarely observed to connect with the adjacent B-subfibre. Fig. 3C, however, illustrates a definite exception to this rule: all of the outer arms bridge the B-subfibres, while inner arms are not apparent. The central matrix region has a mottled appearance, but often in both isolated and intact cilia several diffuse radial linkages are observed within the plane of section (Figs. 2, 3A, C and L). Also the central pair of microtubules are often connected by a single 30 to 40 nm long, arch-shaped bridge, spanning a distance of about 25 nm (Figs. 2, 3E, F and K). The ciliary unit membrane appears tightly applied to the axonemes of cilia *in situ*, whereas in the isolated preparations considerable distortion and fusion of the membranes take place.
Cilia and flagella from a mollusc

The absence of basal bodies and the frequent occurrence of transition zones in fields of isolated cilia and ciliary axonemes (Fig. 1) indicates that in all 3 methods of isolation the process of deciliation takes place by the severance of the cilium from the cell at the junction of the transition zone and the distal end of the basal body (see also fig. 5 of Linck, 1973). Transverse sections through the basal regions of cilia in situ (not shown) indicated that on the proximal side of the basal plate, where the outer fibre doublets acquire the third C-subfibre of the basal body, the skewing of the resulting triplet fibres increases to about 50 degrees (cf. Gibbons & Grimstone, 1960). The separation of the cilia at their transition zones on treatment with the various isolating media may be related to regions of stress in the microtubules existing at these points of increased skewing.

Following 3 extractions of the 2 x-seawater-isolated cilia with 1% Triton X-100, essentially all of the membranes are removed (Fig. 4). The outer doublets retain their cylindrical and skewed configurations, and occasional nexin bridges are observed adjoining 2 sets of doublets. The diameter of the axoneme is usually about 160 nm—about 15% less than in whole cilia. The matrices of the axonemes contain a substantial amount of amorphous material, but occasionally radial linkage fibres are evident (Fig. 4c and f). Frequently one member of the central pair of microtubules is missing. Arms are not readily apparent on the outer A-subfibres in preparations stained with either saturated methanolic uranyl acetate and Reynolds’s lead citrate (Fig. 4A–F) or with the more intensely staining combination of 1% aqueous potassium permanganate and Reynolds’s lead citrate (Fig. 4G–I). In the cases where arms are visible, they are usually observed as single (inner or outer) rather than paired projections. The juxtaposition of the inner arm and the nexin fibre (Fig. 4D) often makes it difficult to distinguish the 2 different structures. Many of the would-be arms are also obscured by an amorphous fuzz which is frequently associated with the outer fibres of the purified ciliary axonemes (e.g. Fig. 4F). Explanations for the difficulty in observing the arms, if present, and the localization of the ATPase will be considered later.

Flagella and flagellar axonemes

During the peak reproductive period (July–August) of *Aequipecten irradians* (Sastry, 1970), ripe gonads were collected for the isolation of sperm flagella. According to the procedure for obtaining sperm and sperm tails outlined earlier, 60 large scallops (6.4–7.6 cm in diameter), containing roughly 100 g of gonads, yield approximately 35 mg of flagellar axoneme protein. The isolated flagella and flagellar axonemes have a specific Mg2+-activated ATPase activity of 0.13 and 0.12 μmol Pj/min/mg protein respectively. The ultrastructure of the intact sperm tails (Fig. 5) and the resulting Triton-extracted flagellar axonemes (Fig. 6) are in general similar to their ciliary counterparts but with certain interesting exceptions. The most noticeable difference is that cross-sections of flagella are considerably clearer, due primarily to the absence of the extensive and amorphous matrix typical of cilia. It is also a common feature in cross-sections of flagellar axonemes for the 9 + 2 structures to appear as C-shaped or fragmented arrays, suggesting greater lability of nexin linkages in flagella than in cilia.
R. W. Linck

after membrane removal. Fragmentation of the axoneme might also result from mechanical disruption during sperm tail isolation, but nevertheless, further studies have verified the greater solubility of the flagellar nexin (Linck, 1973). Another possibility is that 2 of the flagellar outer fibre doublets are unlinked by nexin fibres, leading to the production of the C-shaped arrays, but the occurrence of structures containing fewer than 9 outer fibres would argue against this idea. The disorientation of the flagellar axoneme makes it difficult to determine which, if any, of the outer fibres might be unlinked. Isolated sperm flagella do not contain the basal plates and transition zones common in isolated cilia, since flagella are sheared off distal to these structures during homogenization. Flagellar axonemes are otherwise similar to ciliary axonemes regarding the maintenance of the radial linkage fibres, the doublet skewing, the apparent lack of arms on the A-subfibres and the lability of one member of the central pair of microtubules.

DISCUSSION

A principal contribution of this investigation has been the application of several methods of deciliation to the gills of the lamellibranch mollusc, *Aequipecten irradians*. This species provides high and easily obtained yields of ciliary protein, and also permits the seasonal isolation of sperm flagella, thus making possible comparative studies on cilia and flagella from a single species.

The mechanism by which deciliation takes place is not known; however, the results of treating the gills with twice-concentrated seawater, 10% ethanol–10 mM CaCl₂, or 60% glycerol are essentially the same, namely the rupture of the ciliary membrane at the base of the organelle and the severance of the ciliary axoneme at the junction of the transition zone and the basal body.

Although structurally similar, gill cilia isolated by these 3 methods are physiologically different. Cilia isolated from 2x seawater and from ethanol-calcium do not reactivate in the presence of ATP, yet they have ATPase activities at least as high as glycerol-isolated cilia which do reactivate to some extent (Dr Raymond Stephens, personal communication). Similarly, *Tetrahymena* cilia have been shown previously to reactivate after glycerol treatment but not after treatment with ethanol-calcium (Gibbons, 1965). Ciliary axonemes from 2x seawater and from ethanol-calcium were also noted to have elevated ATPase activities compared to those from glycerol (Table 1).

The presence of an ATPase and the apparent absence of arms in ciliary and flagellar axonemes raise important questions concerning the localization of the axoneme ATPase. Gibbons (1963, 1965a) has demonstrated that purified 30-s dynein obtained from *Tetrahymena* can be reconstituted with ciliary outer fibres, reappearing as the arms on the A-subfibres; on the other hand, no appreciable reconstitution takes place with the 14-s form. In the micrographs of axonemes from *Aequipecten* cilia and flagella, arms are infrequently observed on the A-subfibres, whereas their presence is unequivocal in the micrographs of intact or isolated whole organelles. The apparent lack of arms on either the ciliary or flagellar axonemes, both of which possess extractable
forms of ATPase activity (Linck, 1973), has several possible explanations. The least attractive explanation is that the arms are in fact absent from the enzymically active axonemes, but such a hypothesis cannot be reconciled with the reconstitution experiments of Gibbons (1965a). More likely the arms are present, but they may have become randomly oriented following the removal of the membrane and/or matrix components in such a way that there is a substantial reduction in the electron-density reinforcement arising from successive arms in register. This situation has its analogy in muscle, where cross-bridge patterns during rigor differ greatly from those in the relaxed state (Reedy, 1967, 1968). Allen (1968) has described in cross-sections of intact Tetrahymena cilia the asymmetric nature of the arms and their highly constant orientations about the 9 outer fibres. A range of values has been observed in longitudinally sectioned material for the centre-to-centre spacing of the arms: 13.0 nm in protozoan flagella of termite gut parasites (Gibbons & Grimstone, 1960); 13.5 nm in molluscan gill cilia (Gibbons, 1961a, b); 17.0–20.0 nm in Tetrahymena cilia (see Grimstone & Klug, 1966); and 20.0–22.0 nm for sperm flagella of the blowfly Sarcophaga (Warner, 1970). Assuming that the arms are in perfect register with a longitudinal repeat of 16.0 nm, a transverse section 60.0 nm thick through a cilium or flagellum would show a 3- to 4-fold reinforcement of these structures. If the arms of an axoneme are randomly oriented, i.e. bent in azimuthal or longitudinal directions, a 67–75% reduction would occur in this reinforcement, and considerable resolution would be lost due to the multiple orientations within the section. Some evidence for the flexibility of the arms in Aequipecten gill cilia is given in Fig. 2. In this micrograph of cilia in situ, the arms do not show the highly constant orientations displayed by Tetrahymena, but rather the degree of ‘bend’ is quite variable.

One final consideration concerning the possible flexibility of the arms is relevant here. Summers & Gibbons (1971) have presented substantial evidence for a sliding tubule mechanism of flagellar bending, presumably involving the dynein arms. These authors have suggested that flagellar bending results from cyclic bridge formation, possibly regulated by the degree of separation between the dynein arms of one A-subfibre and the B-subfibre of the neighbouring outer fibre doublet. In the present investigation the specific ATPase activity of ciliary axonemes, isolated from twice concentrated seawater, varied from 0.12 to 0.24 µmol P_i/min/mg protein. Assuming that these variations are not due simply to biological variability, the higher value may represent a 2-fold activation of the specific activity. Increasing concentrations of KCl cause a rise in the ATPase activity of ciliary axonemes (Stephens & Levine, 1970); the converse is true for flagellar axonemes (Gibbons, Fronk & Gibbons, 1970). Unless this phenomenon is irreversible, however, the activation of ciliary axonemes cannot be explained as a salt effect on the enzyme alone, since the ATPase activity of axonemes used in this study was measured in the absence of KCl. Considering that the diameters of isolated ciliary axonemes are approximately 15% less than those of axonemes of cilia in situ, one might speculate that the dynein (arm) activity is activated by its closer proximity to the adjacent B-subfibres.

Cilia and flagella of A. irridians have been observed to differ from one another in their matrix and nexin components. The different responses of the ciliary and flagellar
matrix regions to the process of fixation, embedding and staining, presumably indicate differences in their chemical composition. Some flagellar nexin fibres are absent following membrane removal, leading to the production of fragmented or C-shaped arrays of the 9 + 2 structure. Other differences have now been observed in chemically fractionated cilia and flagella (Linck, 1973), and the effect of these differences in modifying the sliding filament model can now be considered.

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REFERENCES

Cilia and flagella from a mollusc


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Fig. 1. Pellet of cilia isolated from twice-concentrated seawater. The absence of basal bodies and the occurrence of transition zones (tz) in cilia preparations indicate that the organelles separate at this junction. Cilia prepared from ethanol–calcium chloride and from glycerol contain some cell debris, but are otherwise indistinguishable from the above. Stain: uranyl acetate/lead citrate.
Fig. 2. Tangential section through a gill filament of *Aequipecten irradians* showing a field of cilia. Radial link heads (*rlh*) appear as globular subunits midway along the radial linkage fibres (cf. Warner, 1970), and a central sheath (*cs*) bridges one side of the central pair of microtubules. The diameter of the ciliary axoneme is consistently about 190 nm, measured from the centres of the doublet fibres and through the point of least compression. Arrows point to the asymmetric arms of the A-subfibres; pairs of arms may assume either a 'lobo claw' appearance (lower left) or the asymmetric pattern (upper right) described by Allen (1968). The apparent occurrence of clockwise and counterclockwise enantiomorphic forms of cilia (see Gibbons, 1961b) arises from the overlapping of organelles from adjacent gill filaments. Stain: KMnO₄/lead citrate.
Fig. 3. Individual enlargements of cilia isolated from twice-concentrated seawater (A–D), 10% ethanol–10 mM CaCl₂ (E–H) and 60% glycerol (I–L). Structures are well preserved in the isolated organelles: arms (arrows); central sheath (cs); and radial linkage fibres (rt). The cross-sections have been printed with the arms pointing in the clockwise direction; this format will also be used for flagella and for axonemes when it is possible to ascertain their enantiomorphic asymmetry on the basis of the arms or the skewed outer fibres. Arms are usually asymmetrically paired and rarely connected to the neighbouring B-subfibre; however, Fig. 3C represents an interesting exception in which all of the outer arms bridge the 9 doublet outer fibres and none of the inner arms can be seen. Stain: uranyl acetate/lead citrate.
Cilia and flagella from a mollusc
Fig. 4. Ciliary axonemes after extraction with 1% Triton X-100. Only occasional arm structures (arrows) can be seen, usually appearing as single rather than paired projections. Nexin bridges (n) connecting the A-subfibres and radial linkage fibres (rl) are also indicated. Frequently one member of the central pair microtubules is missing. An amorphous fuzz fills the matrix region and is frequently associated with the outer fibres. Note that the diameters of these axonemes are approximately 160 nm, about 15% less than those of cilia in situ (cf. Fig. 3). Stain: A-F, uranyl acetate/lead citrate; G-L, KMnO₄/lead citrate.
Cilia and flagella from a mollusc
Fig. 5. Whole sperm from *Aequipecten irradians* at low (a) and high magnification (b–e), showing arms (arrows), radial link heads (rlh) along the radial linkage fibres, and the central sheath (cs) which bridges the central pair on one side only. A sperm nucleus (nu) and a mitochondrion (m) are also present in Fig. 5a. Stain: a, b, d, KMnO$_4$/lead citrate; c, e, uranyl acetate/lead citrate.
Fig. 6. Sperm flagellar axonemes after extraction with 1% Triton X-100 at low (A) and high magnification (B–G). Partially fragmented and C-shaped 9 + 2 structures are frequently observed, indicating the absence or lability of some of the nexin linkage fibres. The occurrence of arms (arrows) in axonemes is much more infrequent than in whole flagella, whereas radial link heads and radial linkages (r1) and nexin fibres (n) are readily apparent. Stain: uranyl acetate/lead citrate.