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STUDIES ON THE PROTEIN COMPONENTS OF CILIA FROM TETRAHYMENA PYRIFORMIS

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Numerous studies with the electron microscope have established that all cilia and protozoan flagella possess essentially the same fine structure.[1,2] However, interpretation of the function of this structure has been hindered by the fact that little is known about the chemistry of the constituents. ATP can induce motility in glycerinated cilia and flagella,[3,4] and the isolated organelles possess ATPase activity,[4,5] so that the energy for movement presumably comes from dephosphorylation of ATP. Work on the nature of ciliary proteins has been handicapped by their apparent insolubility at neutral pH.[5,6] The experiments reported in this paper demonstrate that the apparent insolubility reported by previous workers is due to the membrane around the cilium, and that after removal of the membrane the remaining protein is soluble in salt solutions at neutral pH. Differential extraction and reconstitution of the ciliary structure, combined with electron microscopy, have made it possible to locate the probable site of ATPase activity. A partial fractionation of the protein components has been accomplished.

Materials and Methods.—*Tetrahymena pyriformis*, strain W, was grown at room temperature in a medium containing 1% peptone, 0.1% yeast extract, and phosphate buffer pH 6.5. The cilia were isolated by a procedure slightly modified from that of Watson and Hopkins,[6] and were stored in a small volume of tris-Mg solution. (Tris denotes tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate. Other abbreviations are those accepted as standard by the *Journal of Biological Chemistry*.)

Except where otherwise stated, all operations were carried out at 0–4°C. Two solutions which were used repeatedly will for convenience be designated as *tris-Mg solution* (2.5 mM $MgSO_4$, 0.03 *M* tris-HCl, pH 8.3 at 0°C), and *tris-EDTA solution* (0.1 mM EDTA, 1 mM tris-HCl, pH 8.3 at 0°C). Routine assays for ATPase activity were carried out by incubating at 20°C in a medium containing 1 mM ATP, 1.2 mM $MgSO_4$, and 0.03 *M* tris-HCl, pH 7.8. Subsequent assay for inorganic phosphate was by the method of Fiske and Subba-Row.[7] Protein assays were

performed by the method of Lowry *et al.*,[8] using a calibration curve prepared with a standard solution of bovine serum mercaptalbumin.[9] Undissolved material was normally removed from suspension by centrifuging at 35,000 *g* for 10 min; substances remaining in the supernatant are, for present purposes, defined as being in solution. Centrifuge pellets to be examined by electron microscopy were fixed with osmium tetroxide, embedded, and sectioned by the usual procedure.[1] Digitonin was obtained from Nutritional Biochemicals Corporation.

Results.—In order to present an intelligible account of the new observations, it is necessary to begin with a brief description of the characteristic fine structure of cilia and flagella; more detailed descriptions have been given elsewhere.[1, 2] The cilia of *Tetrahymena,* like other typical cilia, are approximately cylindrical and composed of a complex bundle of fibers which is embedded in a matrix and enclosed by a membrane (Fig. 1). The principal feature is the backbone of nine double outer fibers and two central fibers that runs continuously along the length of the cilium. Subfiber *A* of each outer doublet bears a double row of projections, called "arms," that extend toward subfiber *B* of the adjacent doublet. Between the outer and central fibers is another set of nine secondary fibers, smaller and less regular than the others. The three groups of fibers are held together at frequent intervals along the cilium by means of radially oriented links.

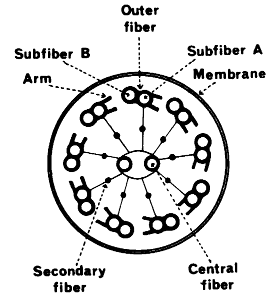


FIG. 1.—Diagram showing the structures visible in a cross section of a cilium. (Modified from ref. 1.)

Examination by light and electron microscopy showed that the preparations of isolated cilia contained no appreciable contamination with cell bodies or cytoplasmic debris. The isolation procedure caused little structural distortion of the cilia (Fig. 2), the only apparent alteration being that the membrane was more closely applied to the bundle of fibers than normal. The cilia could be kept in tris-Mg solution for a few hours without damage, but storage overnight resulted in many of the cilia losing their normal cylindrical shape and rounding up into spheres with the axial fiber bundle coiled around inside the membrane.

Preliminary experiments showed that very little protein was extracted from the

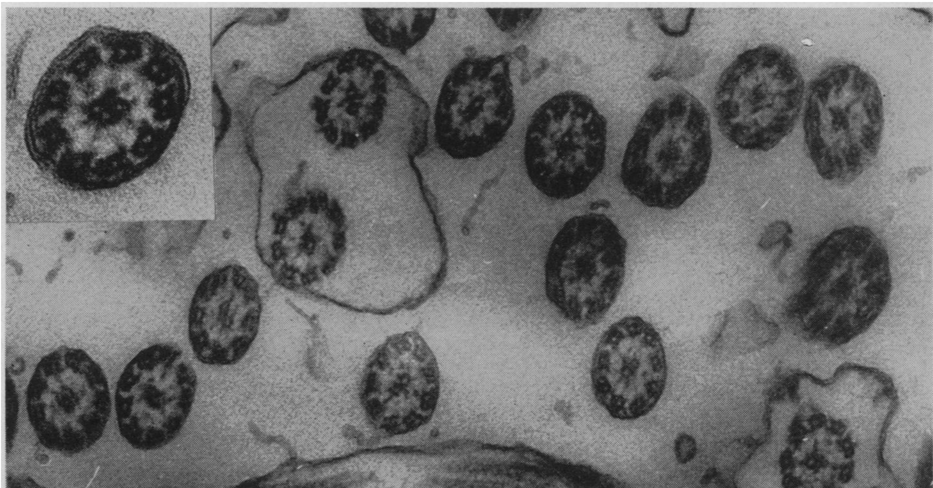


FIG. 2.—Freshly isolated cilia (×65,000). Inset shows detailed cross section (×100,000).

intact cilia by salt solutions at neutral pH. However, certain observations suggested that this apparent insolubility was due to the membrane enclosing the cilium rather than to inherent insolubility of the proteins themselves. Two methods have been used to break down the membrane, as a first step in obtaining the proteins in solution. The method to be described in this paper involves the selective solubilization of the membrane with digitonin, a surface-active glycoside. A second method, which involves disrupting the membrane by dialyzing the cilia against a low concentration of chelating agent, gave comparable results.

The results of extracting freshly isolated cilia twice with a 0.5 per cent solution of digitonin in tris-Mg solution are shown in Table 1. Since the second extraction

TABLE 1

EXTRACTION OF CILIA WITH DIGITONIN

	Total protein (mg)	Total ATPase activity (μmoles P/hr)	Protein in supernatant (mg)	ATPase activity in supernatant (μmoles P/hr)
Freshly isolated cilia in tris-Mg soln. Centrifuged.	4.8	15	0.6 (12%)	—
Pellet resuspended in 0.5% digitonin in tris-Mg soln. Centrifuged.	—	—	1.4 (30%)	2
Pellet resuspended in 0.5% digitonin in tris-Mg soln. Centrifuged.	—	—	0.3 (6%)	—
Pellet resuspended in tris-Mg soln.	2.4 (50%)	14	—	—

Figures in parenthesis represent percentages of initial protein.

with digitonin brings much less protein into solution than the first, it is reasonable to regard the ciliary protein as divisible into two fairly well-defined fractions, one soluble and one not soluble in digitonin under these conditions. The insoluble residue remaining after the second extraction will be called *digitonin-extracted cilia*, and the protein composing it will be called *structural protein*. The structural protein, which contains almost all the fibrillar ATPase activity, constitutes about 50 per cent of the total ciliary protein. When the pellet of digitonin-extracted cilia is examined by electron microscopy, the principal change apparent is that the membrane has been completely removed (Fig. 3). In most other respects the structure of the cilium appears to be intact and unaffected by the extraction. All the nonmembranous components shown in Figure 1 are still present, although slight indications of breakdown, such as a missing central fiber or a damaged outer fiber, can be seen in some cilia.

The structural protein of the cilium is almost wholly soluble in moderately concentrated solutions of salt. For example, a suspension of digitonin-extracted cilia in 0.6*M* KCl becomes gradually less turbid over a period of several hours as the protein goes into solution; after 18 hr 90–95 per cent of the protein and ATPase activity are in solution (Table 2), and centrifugation yields only a very small pellet composed mostly of residual lipid. The solution of structural protein has a typical protein absorption spectrum with a maximum near 276 mμ, and an absorbancy ratio 280/260 mμ of 1.3. Suspension in 0.6 *M* KI likewise brings almost all the protein into solution, but in this case the action appears instantaneous.

A useful fractionation of the structural protein is obtained by treating with a chelating agent at very low ionic strength. Dialysis of the digitonin-extracted cilia against tris-EDTA solution brings almost all the ATPase activity into solu-

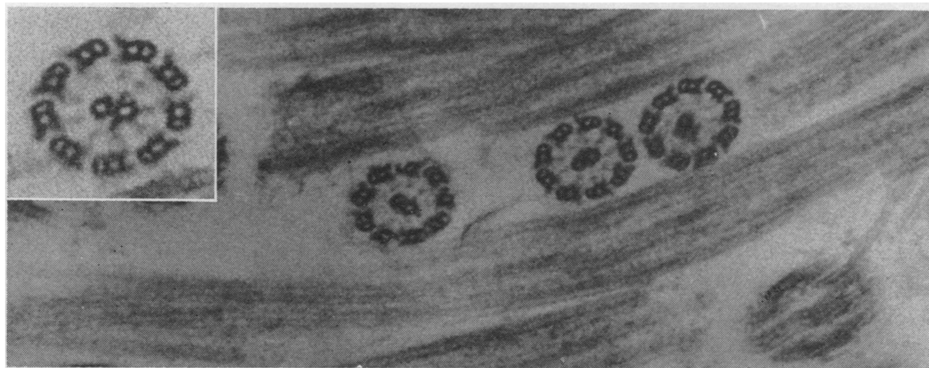


Fig. 3.—Digitonin-extracted cilia (×65,000). Inset shows detailed cross section (×100,000).

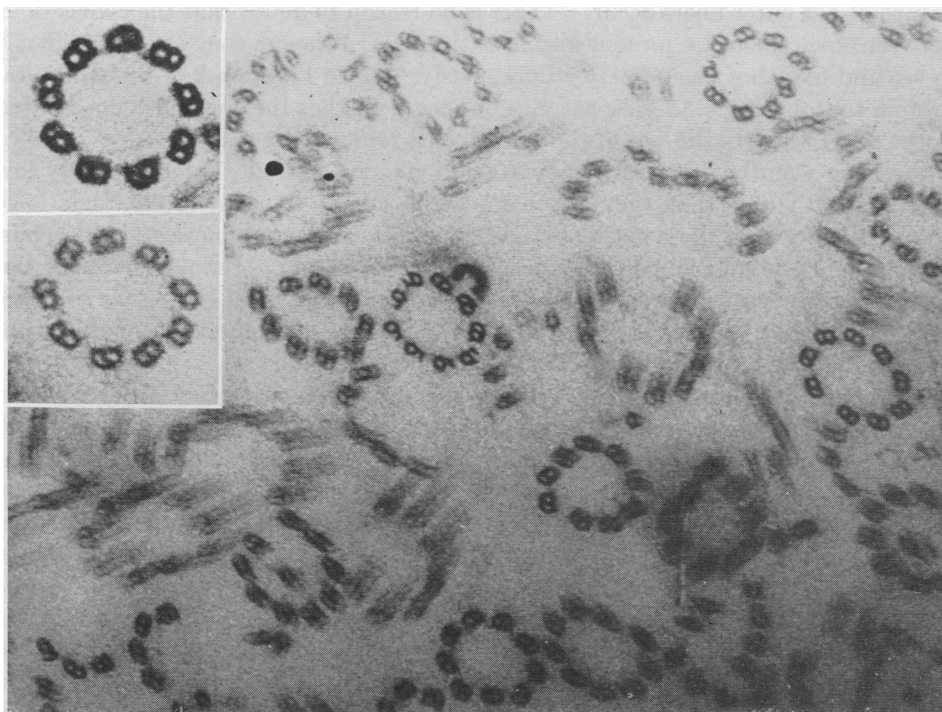


Fig. 4.—The insoluble residue after dialyzing digitonin-extracted cilia against tris-EDTA solution (×65,000). Inset shows detailed cross section (×100,000).

TABLE 2

Solubility of Digitonin-extracted Cilia at pH 8.3

Extracting solution	Time of extraction	Per cent protein in solution	Per cent activity in solution	Specific activity of solution, μmoles P/(mg protein × hour)
0.6 *M* KCl, 30 mM tris-HCl, 2 mM $MgSO_4$	18 hr	95	90	5.3
1 mM tris-HCl, 0.1 mM EDTA	Dialyzed 18 hr	31	90	20
1 mM tris-HCl, 1 mM $MgSO_4$	18 hr	7	4	—
10 mM KCl, 30 mM tris-HCl, 1 mM EDTA	Dialyzed 18 hr	6	2	—
3 mM ATP, 30 mM tris-HCl, 2.5 mM $MgSO_4$	5 min	5	—	—

tion, but only about 30 per cent of the protein (Table 2). The soluble fraction obtained by this dialysis will be denoted *Fraction 1* of the structural protein; the residue, insoluble in tris-EDTA solution, will be called *Fraction 2*. Examination of this insoluble residue in the electron microscope shows it consists of the outer fibers alone (Fig. 4); the other structural components, including the arms on the outer fibers, have been almost completely removed. It is remarkable that the arrangement of outer fibers found in an intact cilium still largely persists. Thus, many of the outer fibers occur in sets of nine, grouped around the periphery of a cylinder 0.2 μ in over-all diameter; even details of their arrangement such as their slight inward twist are maintained. It seems that the outer fibers are held together in these cylindrical groups by means of a thin filament running around the inside of the cylinder from one A subfiber to the next. Not all the cylinders in a preparation are intact; frequently the wall is broken open at one place, and sometimes smaller groups of 1–8 outer fibers occur. There is no reason to doubt that the connections between A subfibers are present also in native cilia, although they are very difficult to see and have not been described previously. Since Fraction 2 of the structural protein contains little ATPase activity, it is evident that the enzyme is not located in the outer fibers but in one of the components removed by dialysis.

We have seen that the digitonin-extracted cilia are partially soluble at very low ionic strength and that they are completely soluble at moderately high ionic strength. Between these extremes, however, the structure is more stable. Thus, 1 mM $MgSO_4$ is sufficient to maintain it intact (Table 2). Divalent ions are not essential, however, for the structure is also stabilized by 10 mM KCl in the presence of EDTA. Only a small amount of protein goes into solution when the digitonin-extracted cilia are treated with ATP.

It seemed of interest to see whether any reconstitution of the fine structure could be obtained by restoring magnesium to the dialyzed preparation. In one such experiment, a preparation of digitonin-extracted cilia was dialyzed against tris-EDTA solution and then divided into two. To one half, tris buffer and $MgSO_4$ were added to make the concentrations up to the standard tris-Mg solution; the other half of the preparation was left in tris-EDTA solution as a control. After 90 min, the two were centrifuged, the supernatants assayed for ATPase activity and for protein, and the pellets fixed for electron microscopy. In the preparation to which magnesium had been restored, 17 per cent of the total protein and 42 per cent of the total activity were in solution, while in the control 31 per cent of the protein and 88 per cent of the activity were in solution. These results show that about half of the protein and half of the activity which went into solution on dialysis have become re-bound to the insoluble residue. Electron micrographs of the pellets from this same experiment are shown in Figures 4 and 5. The principal structures to have returned in the reconstituted preparation are the arms on the outer fibers. These appear to have returned with a remarkable degree of precision to the same position that they had in the intact cilia, with one pair of arms located on subfiber A of each outer fiber (Fig. 5). It is not yet possible to say what percentage of the arms has returned on reconstitution, partly because their distribution along the length of the outer fibers is difficult to determine. The precision with which the arms return illustrates the morphogenetic potential inherent in the recombining units and is relevant to the question of how cilia are formed. In addition to the

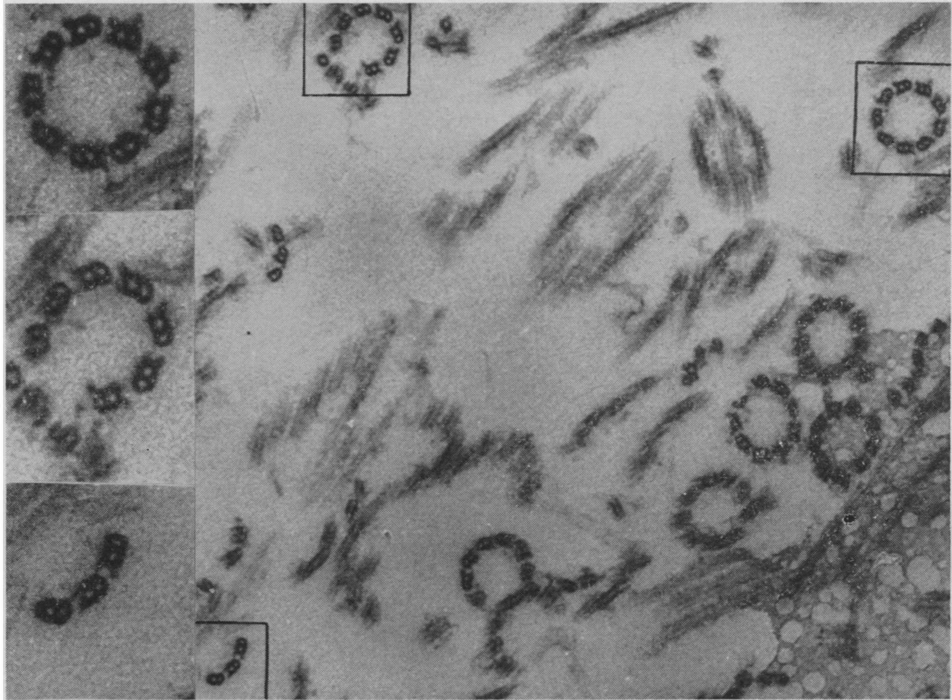
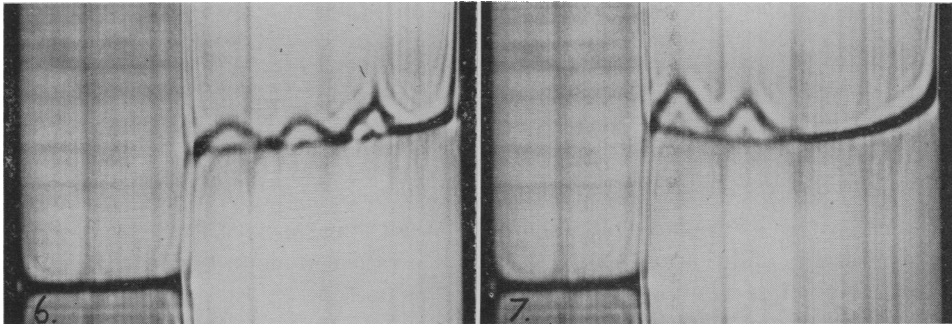


FIG. 5.—"Reconstituted" cilia obtained by restoring magnesium to a preparation of digitonin-extracted cilia that had been dialyzed against tris-EDTA solution (×50,000); insets show portion of same micrograph (×100,000). (The same preparation of dialyzed cilia but without addition of magnesium is shown in the upper inset of Fig. 4.)



FIGS. 6 AND 7.—Analysis by ultracentrifugation of Fraction 1 of structural protein. Two cells were run, one containing sample, the other a solvent blank. Figure 6 shows Fraction 1 in tris-EDTA solution. Figure 7 shows the same preparation of Fraction 1 in tris-EDTA solution containing 0.1 M Na_2CO_3. Kel-F centerpiece; 59,780 rpm; 20°C; bar angle, 50°; time: 16 min after reaching full speed.

structures which return specifically to their original position, there is also some nonspecific adsorption. This appears as "fluffy" material coating the outer fibers.

The above evidence indicates that the presence or absence of ATPase activity in the ciliary residue is correlated with the presence or absence of the arms on the outer fibers. The correlation strongly suggests that the arms are the site of at least part of the fibrillar ATPase activity of the cilium (see Discussion).

Analysis by ultracentrifugation, and further purification of the ATPase: When Fraction 1 of the structural protein is examined by analytical centrifugation, three principal peaks are visible with approximate sedimentation coefficients ($s_{20,w}$) of 4, 13, and 25 S (Fig. 6), together with a minor peak at about 45 S. The 4 S and 13 S peaks are symmetrical, but the fastest (25 S) peak frequently has a trailing shoulder indicating the presence of some material sedimenting in the range 20–25 S. As the 25 S peak crosses the field, it appears to spread rather more slowly than the other two peaks, and it bears a characteristic hypersharp tuft on its crest; these properties suggest a degree of asymmetry in the 25 S particles. The relative area of the 13 S and 25 S peaks appears to vary somewhat in different preparations of Fraction 1. In the preparation shown in Figure 6, for example, the two have approximately the same area, but in some other preparations the 13 S peak was much smaller relative to the 25 S peak. The factors responsible for this variation are not yet known. Substantially the same sedimentation pattern is seen whether the Fraction 1 is run in tris-EDTA solution, in tris-EDTA solution containing 0.1 *M* KCl, or in tris-Mg solution. Addition of 3×10^{-3} *M* ATP to the sample in the centrifuge cell immediately before a run in tris-Mg solution had no effect on the pattern.

The variation in the relative size of the 13 S peak suggested that it might be a breakdown product of the 25 S peak. This hypothesis was confirmed by running Fraction 1 in alkali (0.1 *M* Na_2CO_3) instead of at the usual pH 7.8;[10] then only two peaks sedimenting at about 3.5 S and 11 S are seen (Fig. 7). These two peaks probably correspond to the 4 S and 13 S peaks of the usual pattern, the slight decrease in sedimentation constant being due to the difference in pH. Comparison of area under the peaks shows that the total area is about the same at both pH's, with the 25 S peak in Figure 6 being about equally divided between the 4 S and 13 S peaks in Figure 7. As the same preparation of Fraction 1 was used, this result clearly shows that treatment with alkali breaks down the 25 S particles to a mixture of 4 S and 13 S. However, the factors determining the presence of both 13 S and 25 S particles in all the normal preparations are still unknown.

A solution of Fraction 2 in 0.6 *M* KCl showed a single peak sedimenting at about 4 S, together with a large amount of heterogeneous material sedimenting in the range 4–50 S. A solution of whole digitonin-extracted cilia in 0.6 *M* KCl showed the same pattern as would be obtained by adding the patterns of Fractions 1 and 2 run separately; addition of 3×10^{-3} *M* ATP to the sample in the centrifuge cell immediately before running had no effect on this pattern.

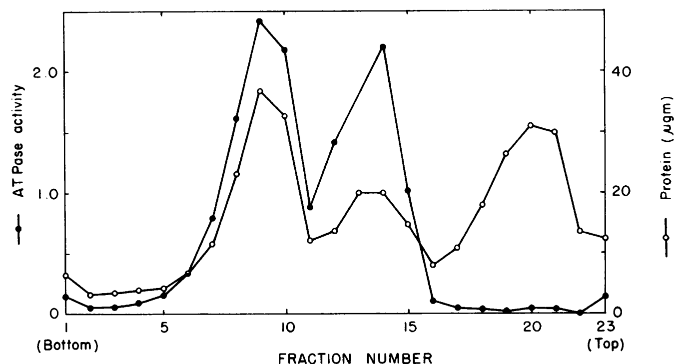


FIG. 8.—Separation of the components of Fraction 1 by centrifugation through a sucrose density gradient. 0.1 ml of Fraction 1 in tris-EDTA solution layered on top of a 5–30% sucrose gradient, and centrifuged 7 hr at 38,000 rpm in SW 39 rotor of Spinco centrifuge. Contents of centrifuge tube collected in samples of three drops each. ATPase activity in μmole P/hour. No activity measurement sample 13.

The three principal components of Fraction 1 have been separated by zonal centrifugation through a sucrose density gradient.[11] Analysis for protein showed that the 4, 13, and 25 S components were well resolved (Fig. 8). All three components had typical protein absorption spectra with maxima near 280 mμ and absorbancy ratios 280/260 mμ of 1.3–1.4. The ATPase activity was about equally divided between the 13 S and 25 S components; the 4 S was completely inactive (Fig. 8). The specific activities of the 13 S and 25 S components were 77 and 40 μmole P/(mg protein $\times$ hour), respectively (average of four experiments). Taken with the other evidence above, the higher specific activity of the 13 S component indicates that it is the enzymatically active portion liberated on breakdown of the 25 S particles. The 13 S component is the most highly purified form of the fibrillar ATPase prepared so far. It has a specific activity about 15 times higher than that of whole cilia, and represents about 8 per cent[12] of the total ciliary protein.

Discussion.—Now that the ciliary proteins can be obtained in solution under mild conditions they become amenable to study by standard physical-chemical methods. However, it should not be assumed that one is necessarily dealing with true monomeric molecules, for the very fact that the proteins occur aggregated together in the structure of the cilium suggests that similar, although smaller, aggregates and complexes may also occur in solution. It is not yet known how many distinct proteins are present in the structural protein fraction. The experimental data so far require at least two (one with ATPase activity and sedimenting at about 13 S, the other with no ATPase activity and sedimenting at about 4 S), but there may well be more. The 25 S particles found in solutions of structural protein probably represent a naturally occurring subunit of the cilium, one which is partially broken down in the normal course of preparation, and which is wholly broken down to a mixture of 4 S and 13 S particles by 0.1 M Na_2CO_3.

When studying the chemistry of cilia, it is customary to look for analogies with the chemistry of muscle. The properties of Fraction 2 of the structural protein have some likeness to those of actin, while the ATPase of cilia appears to resemble the ATPase of the mitotic apparatus[13] more than it does that of myosin. However, the value of comparisons based on such fragmentary evidence is open to question.

Current hypotheses usually assume that the bending movements of cilia are the result of the localized shortening of longitudinal contractile elements,[1, 14] and the experiments reported here are consistent with a mechanism of this type. Since the immediate source of energy for motility is provided by dephosphorylation of ATP, it seems probable that the mechanical force responsible for motility must be initially generated within monomolecular distance of the site of ATPase activity. This suggests that the longitudinal elements which contract are the outer fibers and that the contractions result from the interaction of the arms with two adjacent outer fibers. The forces resulting from a contracted region of one outer fiber would thus be transmitted automatically to the adjacent outer fibers on each side, and this would account for the spread of contractions around the cilium which is necessary to account for the observed bending movements.

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