# SOME PROPERTIES OF THE PROTEIN

# FORMING THE OUTER FIBERS OF CILIA

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ABSTRACT

Cilia were isolated from *Tetrahymena pyriformis* by an ethanol–calcium procedure. Solutions of outer-fiber protein were obtained either by aqueous extraction of an acetone powder of whole cilia, or by dissolving the isolated outer-fibers in 0.6 M KCl. In aqueous solution, the outer-fiber protein has a sedimentation coefficient of 6.0S and a molecular weight of 104,000 ± 14,000. In 5 M guanidine hydrochloride solution the molecular weight falls to 55,000 ± 5,000. After reduction and alkylation in 8 M urea, about 95% of the protein migrates as a single band on electrophoresis in polyacrylamide gel at pH 8.9; the migration velocity is identical with that of reduced and alkylated actin. Freshly prepared outer-fiber protein contains about 7.5 sulfhydryl groups per 55,000 g of protein. The amino acid composition of outer-fiber protein resembles that of actin, with such differences as occur being of the same order as those between actins from different species of animal.

It has recently become possible to fractionate the principal structural components of cilia (14, 15), and to obtain them in a pure form suitable for study by the usual methods of protein chemistry. In this paper we report a partial characterization of the protein forming the outer fibers of cilia from *Tetrahymena pyriformis*.

The outer-fiber protein appears to be very closely related to the colchicine-binding protein demonstrated by Borisy and Taylor (4) in a variety of tissues rich in cytoplasmic microtubules. In many respects, the properties of the outer-fiber protein also resemble those of the muscle protein, actin. However, work published elsewhere (38) demonstrates that the outer-fiber protein contains a bound guanine nucleotide unlike actin which contains adenosine diphosphate.

Preliminary reports of this work have been published previously (12, 29).

## MATERIALS AND METHODS

Cilia were isolated from *Tetrahymena pyriformis* by the modified ethanol–calcium procedure described in detail elsewhere (14, 43). Three different procedures have been used to extract the outer-fiber protein from the isolated cilia.

### Procedure 1

This consists of making an acetone powder of whole cilia, and extracting the powder with buffer of low ionic strength. The cilia are first washed with Tris–EDTA-30 mM KCl solution (1 mM Tris–HCl buffer, pH 8.3 at 0°C, 0.1 mM EDTA, 30 mM KCl). Approximately 25 mg of the washed cilia are extracted twice with 5 ml of acetone at 0–5°C in a glass tube. The suspension is centrifuged, and the pellet is dried. The resultant powder can be stored over silica gel at −20°C for at least a month with no great deterioration.

The outer-fiber protein is extracted from the powder

in the following manner. Approximately 25 mg of powder are resuspended, with stirring, in 0.5 ml of 1 mM Tris–HCl buffer, pH 7.7 at 0°C, for 30 min. The mixture is then centrifuged, the supernatant is saved, and the extraction is repeated. The supernatants are combined and spun for 10 min at 35,000 g to get rid of contaminating particles. Approximately 30% of the total protein goes into solution.

## Procedure 2

The isolated cilia are resuspended in Tris–EDTA solution (1 mM Tris–HCl buffer, pH 8.3 at 0°C, 0.1 mM EDTA), at a concentration of 5–10 mg per ml, dialyzed against 100 volumes of this same solution for 24–48 hr, and then centrifuged. The matrix protein, the central fibers, and most of the dynein, the ATPase protein associated with the outer fibers, remain in the supernatant. The pellet consists largely of a mixture of ciliary membranes and outer fibers (Fig. 1). This pellet fraction is resuspended in Tris–EDTA solution, and dialyzed for a further 24 hr to get rid of residual dynein. The suspension is then centrifuged, and the pellet is resuspended twice in 0.5% digitonin, 1 mM Tris–HCl, pH 8.3 at 0°C, followed by two washes with plain Tris buffer. The digitonin solubilizes the membranes, and a pure outer-fiber fraction is obtained (Fig. 2). This fraction can then be solubilized directly by resuspending in Tris–EDTA–0.6 M KCl solution (0.6 M KCl, 10 mM Tris–HCl buffer, pH 8.3 at 0°C, 1 mM EDTA). Although 0.6 M KCl is necessary to solubilize the fibers, the protein does not precipitate when the KCl is subsequently removed by dialysis against dilute tris buffer. About 30–40% of the total ciliary protein is recovered in this outer-fiber fraction.

## Procedure 3

Alternatively, the outer-fiber and membrane fraction (Fig. 1) is resuspended in Tris–EDTA–0.6 M KCl solution, and dialyzed against this solution overnight. The outer fibers are solubilized, and the membranes are left as an insoluble residue.

## Reduction and Alkylation

Protein samples were reduced and alkylated by a modification of the method of Crestfield et al. (8). For reduction, the protein solution was made 0.12 M in mercaptoethanol and 8 M in urea (in that order) and dialyzed against 50–100 volumes of reducing solution (0.12 M mercaptoethanol, 8 M urea, 0.1% EDTA, 0.35 M Tris–HCl buffer, pH 8.8 at 20°C) for 12–24 hr at room temperature. 9 volumes of the reduced protein solution were then mixed with 1 volume of alkylating solution (1.1 M sodium iodoacetate, 8 M urea). The mixture, which now contains equimolar amounts of mercaptoethanol and iodoacetate, was allowed to stand at room temperature

for about an hour in the dark and then was dialyzed against 50–100 volumes of 8 M urea, 30 mM Tris–HCl, pH 7.8, also in the dark. In some preparations, the reduction and alkylation were carried on at pH 9.5, with glycine buffer rather than Tris, in order to ensure complete reaction of the sulfhydryl groups.

## Electrophoresis

Disc electrophoresis was performed with poly-acrylamide gels made with 8 M urea according to the procedure of Davis (9). On occasion, the gels were polymerized with riboflavin instead of ammonium persulfate as a catalyst (5). The pH of the sample and spacer gels was 6.7, while that of the running gel was 8.9. The final acrylamide concentrations in the sample, spacer, and running gels were 2.5, 2.0, and 7.5%, respectively. Runs were carried on for approximately 1 hr at room temperature, after which the gels were fixed with 7.5% acetic acid and stained with amido Schwartz. About 50 $\mu$g of protein was loaded per tube.

## Molecular Weight Determinations

These determinations were made by means of the Archibald, sedimentation–diffusion, and sedimentation–equilibrium methods. All runs were carried out at 20°C. The solvents employed were guanidine hydrochloride (5 M guanidine hydrochloride, 0.12 M mercaptoethanol) and Tris–HCl (1 mM Tris–HCl buffer, pH 7.5 at 20°C, with the occasional addition of 0.2 mM GTP).

The multispeed method of Mueller was employed for the Archibald experiments (26), with approximately 0.35 ml of sample loaded in a double sector cell. The speed of the run was increased stepwise from 8225 to 14290 rpm for samples in Tris–HCl, and from 17,250 to 29,450 rpm for samples in guanidine hydrochloride. A six-channel Yphantis cell was used for the sedimentation–equilibrium technique, which made it possible to run three different protein concentrations simultaneously. A sample volume of approximately 50–60 $\mu$l was used, which gave a column height of approximately 1.5 mm. Approximately 25 $\mu$liters of FC43 fluorocarbon were layered at the bottom of each sample compartment. Equilibrium was reached within 5–7 hr for the samples in Tris at 14,290 rpm, and overnight for the samples in guanidine at 15,220–21,740 rpm. Results were plotted in the form $\log\left(\frac{1}{x} \cdot \frac{dc}{dx}\right)$ versus $x^2$, where

$x$ is distance from center of rotation, and $\frac{dc}{dx}$ is the concentration gradient (33). The diffusion coefficient was measured by means of a low-speed run with a synthetic boundary cell (32).
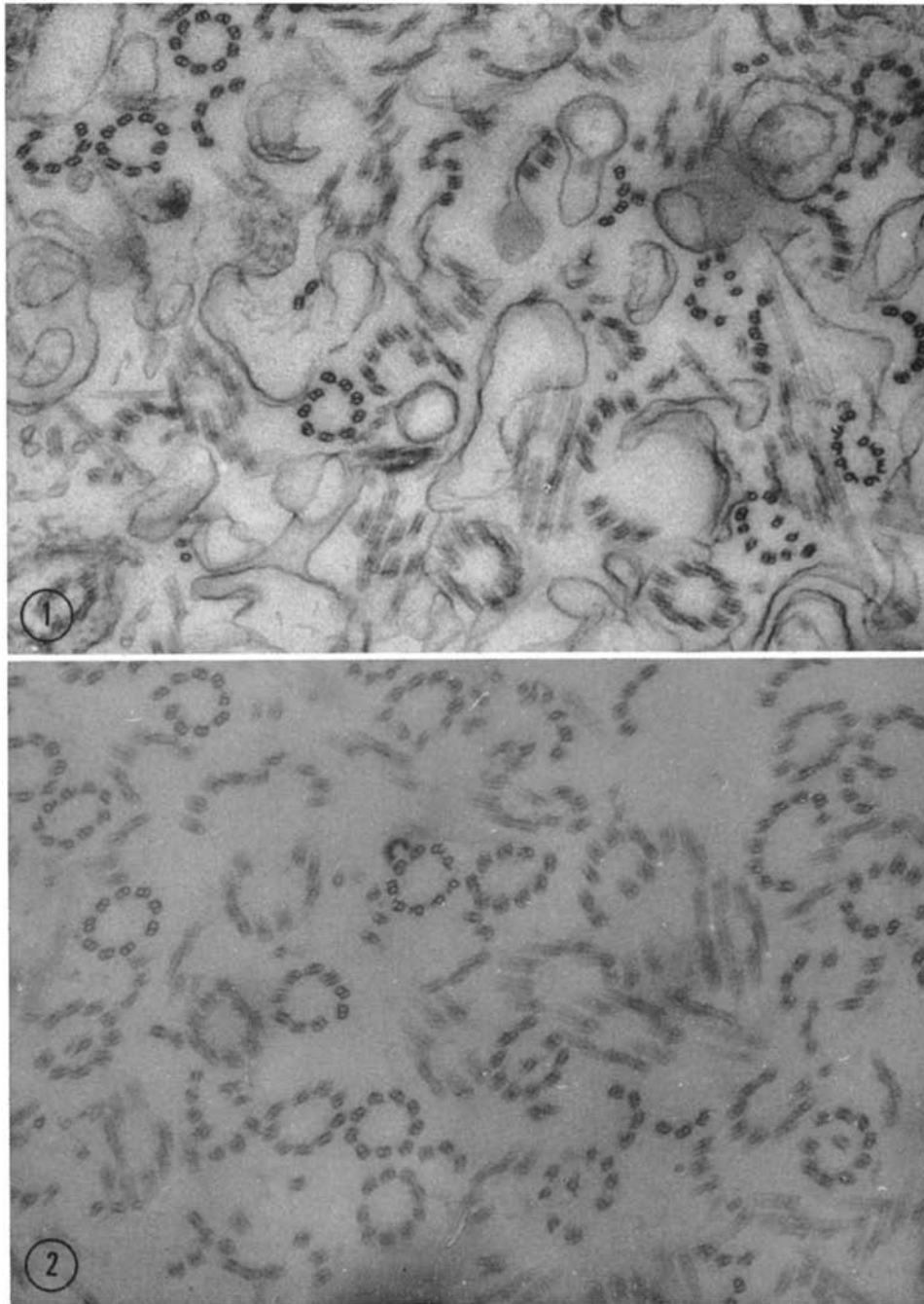
FIGURE 1   Outer-fiber and membrane fraction obtained from cilia after dialysis against Tris–EDTA. × 47,000.

FIGURE 2   Outer-fiber fraction obtained from cilia after dialysis against Tris-EDTA and extraction with 0.5% digitonin. × 56,000.

## Amino Acid Analysis

For amino acid analysis, three equal aliquots, each containing 2–3 mg of protein, were hydrolyzed in 6 N HCl at 110°C (24) for approximately 24, 48, and 72 hr, respectively. The amounts of serine and threonine were corrected by extrapolation to zero hydrolysis time. Tyrosine and tryptophan were determined from the absorbance at 280 and 294.4 m$\mu$ of protein solutions of known concentrations dissolved in 0.1 N NaOH (2); readings taken between 330 and 410 m$\mu$ showed that the amount of scattering was negligible. Cysteine was determined by reaction with DTNB (dithio-bis-nitrobenzoic acid) (10). An aliquot of a protein solution of known concentration was added to the DTNB solution (0.1 mM DTNB, 1 mM EDTA, 60 mM Tris–HCl buffer, pH 7.8 at 20°C), and the progress of the reaction was followed by measuring the change in absorbance at 412 m$\mu$ with time. After the absorbance had reached a steady value (30–45 min), the solution was made 8 M in urea, and an additional reading was taken after 10 min. It was

necessary to have a blank to which the urea is also added. For purposes of calculation, all the measured absorbances were corrected to a standard volume, by assuming Beer's law to be valid.

## Materials

All the chemicals employed were of reagent grade, and the solutions were all made with deionized water. Urea was recrystallized once from 95% ethanol. Iodoacetic acid was recrystallized once from deionized water. The guanidine hydrochloride was prepared from guanidine carbonate (20).

## RESULTS

All the fractions obtained from cilia by procedures 1, 2, and 3 contain the same major protein component, as is evidenced by the similarity in electrophoretic patterns described below, and by the fact that each of them has about the same percentage of the total ciliary protein. However, the
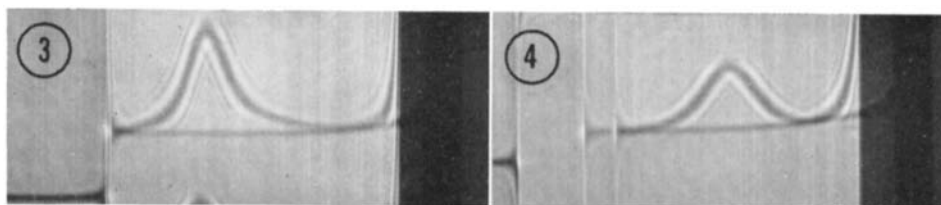


FIGURE 3   Ultracentrifugation of outer-fiber protein obtained from procedure 1, in 1 mM Tris–HCl, 0.2 mM GTP; 48 min after reaching speed of 47,660 rpm; bar angle 60°; concentration, 7 mg/ml.
FIGURE 4   Ultracentrifugation of outer-fiber protein prepared by procedure 3, in 5 M guanidine hydrochloride, 0.12 M mercaptoethanol.
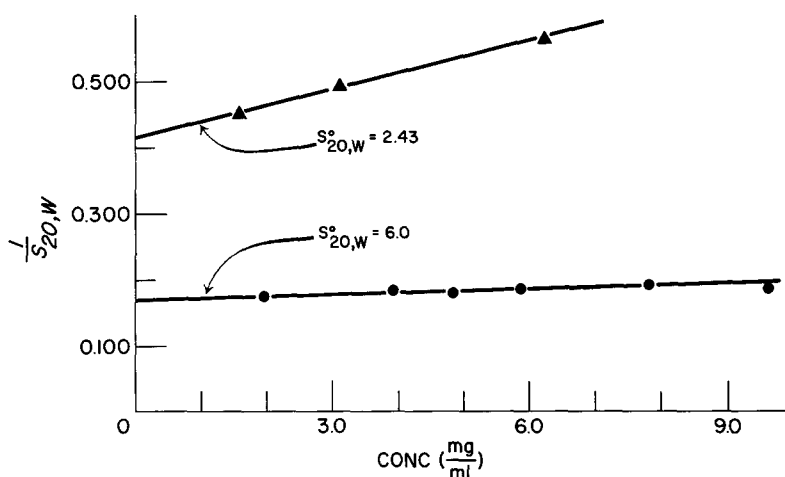


FIGURE 5   Variation of the sedimentation coefficient with concentration of outer-fiber protein. Values obtained by extrapolation to zero concentration are indicated by arrows. Triangles indicate outer-fiber protein in 5 M guanidine hydrochloride, 0.12 M mercaptoethanol; circles indicate outer-fiber protein in 1 mM Tris–HCl.
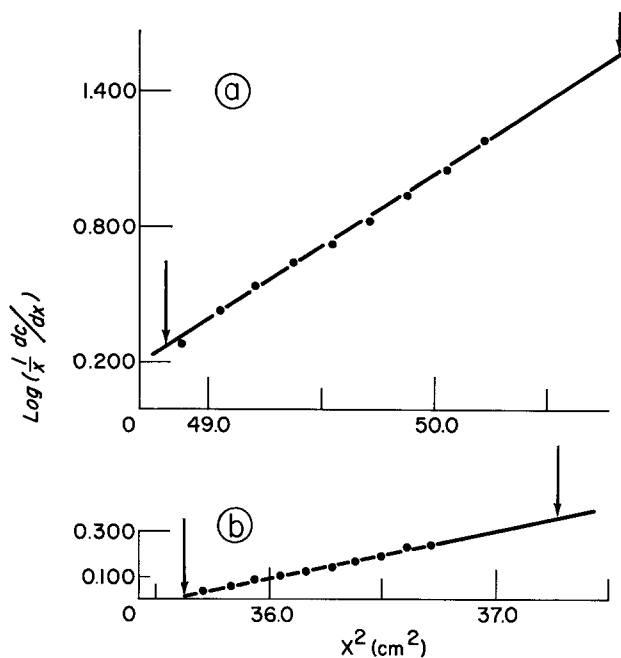
**FIGURE 6** Plot of sedimentation-equilibrium data ($X$ = distance from the center of rotation; $dc/dx$ = protein concentration gradient). Arrows indicate the position of the air and the oil menisci. $a$, Outer-fiber protein from procedure 1, 2.8 mg/ml, in 1 mM Tris–HCl, 0.2 mM GTP. $b$, Same as $a$, but in 5 M guanidine hydrochloride, 0.12 M mercaptoethanol.

TABLE I

*Molecular Weight of Outer-Fiber Protein*

| Method | No. of preparations | Molecular weight* |
|---|---|---|
| Protein from procedure 1 in 1 mM Tris–HCl | | |
| Sedimentation equilibrium | 3 | 103,000 ± 14,000 |
| Protein from procedures 1–3 in 5 M guanidine hydrochloride, 1% mercaptoethanol | | |
| Archibald | 6 | 55,200 ± 2,600 |
| Sedimentation-equilibrium | 3 | 49,300 ± 1,400 |
| Sedimentation-diffusion | 5 | 57,000 ± 5,400 |
| Protein from procedure 1, reduced and alkylated in 5 M guanidine hydrochloride | | |
| Archibald | 1 | 58,800 |

\* Partial specific volume was assumed to be 0.72.

physical state of the protein differs, and only procedure 1 gives sufficiently monodisperse solutions to be suitable for direct study by physical techniques. The solutions obtained by procedures 2 and 3 are highly aggregated, and so are less well suited to physical study; their chief importance is that they permit the identification of the protein with the outer fibers.

*Analytical Ultracentrifugation*

Protein from procedure 1 sediments as a fairly symmetrical peak with a sedimentation coefficient ($S_{20,w}$) of 6.0S, when extrapolated to zero concentration (Figs. 3 and 5). This sedimentation coefficient shows little dependence on concentration. The presence of small amounts of material sedimenting both faster and slower than the 6S principal component is indicated by the slight leading and trailing shoulders on the peak. Preliminary experiments suggest that the amount of leading and trailing material is decreased when the solution used to extract the acetone powder contains 0.2 mM GTP, but this needs to be confirmed by further work.

Solutions obtained by procedures 2 and 3 show a broad peak which spreads rapidly and practically disappears before it has moved halfway down the cell. This pattern indicates that the bulk of the protein is present as large heterogeneous aggregates that sediment in the range 4–30S (12, 14). The homogeneity of these solutions was not improved on removing the KCl by dialysis against 1 mM tris buffer. To disperse the aggregates in these preparations, we have resorted to adding guanidine hydrochloride.

Solutions of outer-fiber protein containing 5 M guanidine hydrochloride and 1% mercaptoethanol (v/v) appear homogeneous in all cases. On centrifugation, one obtains a single symmetrical peak with a sedimentation coefficient $(S_{20,w})$ of 2.25 ± 0.2S, extrapolated to zero concentration (Figs. 4 and 5). The breadth of the peak and the value of the sedimentation coefficient were not affected by the particular procedure used to obtain the protein.

All the molecular weight determinations in guanidine hydrochloride solution by means of the Archibald, sedimentation–diffusion, and sedimentation–equilibrium methods agree on a molecular weight of 55,000 ± 5,000 (Figs. 6–8, Table I).

Similar results were obtained with protein that had been reduced and alkylated. The apparent value of the molecular weight depended appreciably upon protein concentration, and it was essential to extrapolate to zero concentration to obtain a significant result. Since 5 M guanidine hydrochloride effectively breaks most noncovalent interactions and disperses nearly all proteins to their fundamental polypeptide subunits (40), the value of 55,000 probably represents the molecular weight of the peptide chain of outer-fiber protein. The possible binding of guanidine to the protein, and a change in partial specific volume might result in a systematic error of 5–10% in this value.

In the absence of guanidine hydrochloride, only the protein from procedure 1 is homogeneous enough to permit a significant measurement of molecular weight, and even in this case the slight lack of homogeneity leads to some complications. The most satisfactory results were obtained with the sedimentation–equilibrium technique, in which the homogeneity of different preparations could be tested by the linearity of plots of log $\left( \frac{1}{x} \cdot \frac{dc}{dx} \right)$ versus $x^2$. Protein in 1 mM tris buffer from
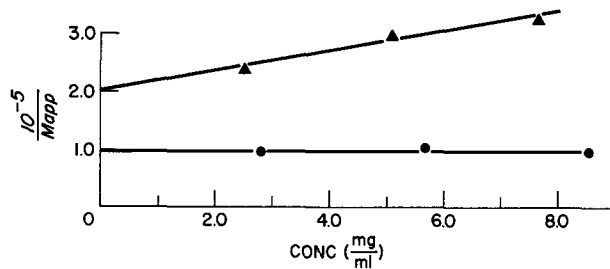


Figure 7 Concentration dependence of the apparent molecular weight obtained by sedimentation-equilibrium, for the outer-fiber protein (procedure 1). Triangles represent protein solubilized in 5 M guanidine hydrochloride, 0.12 M mercaptoethanol; circles represent protein in 1 mM Tris–HCl. When the values are extrapolated to zero concentration, molecular weights of 50,000 and 103,000 are obtained respectively.
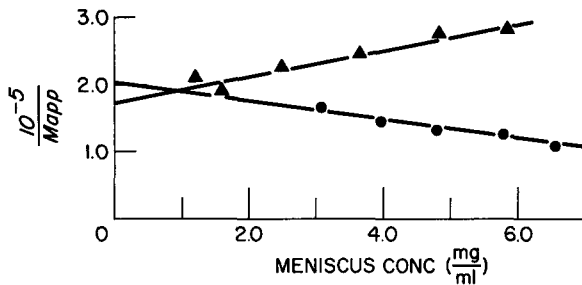


Figure 8 Variation of apparent molecular weight with meniscus concentration during an Archibald run. Triangles represent outer-fiber protein in 5 M guanidine hydrochloride, 0.12 M mercaptoethanol. The circles represent protein from procedure 1 in 1 mM Tris–HCl. Extrapolation to zero concentration gives molecular weights of 56,000 and 49,000 respectively.

procedure 1 gave a fairly straight line in such plots, with some upward curving. The most homogeneous preparations were obtained when 0.2 mM GTP was added to the extraction medium, in which case a good straight line was obtained (Fig. 6). The molecular weight did not depend appreciably upon protein concentration. The results varied between 87,000 and 120,000 in different preparations, with a mean value of 104,000 ± 14,000. Attempts to use the Archibald technique on these preparations gave contradictory results in which the apparent value of the molecular weight at the meniscus decreased as the meniscus concentration decreased during the course of the run. Extrapolation to zero meniscus concentration gave a value of about 50,000. We believe this discrepancy to result from the presence of material both heavier and lighter than the 6S principal component. During the run, the meniscus will be preferentially depleted of the heavier components, so that extrapolation to zero concentration will tend to give the molecular weight of the lightest component. We conclude that the molecular weight of the 6S principal component is close to the value of 104,000 ob-

tained by sedimentation equilibrium, and that the 6S particle is a dimer formed by two of the 55,000 molecular weight polypeptide chains. Small amounts of the 55,000 monomer and of higher aggregates are also present in these solutions.

## Electrophoresis

In our experience, electrophoresis of unmodified outer-fiber protein has been unsatisfactory, because much of the protein is retained in the sample gel and because what does migrate does so in the form of broad, diffuse bands. A more satisfactory pattern is obtained when the protein is reduced and alkylated prior to the run. In this case the sample gel appears completely clear, and only very small amounts of protein are retained at the interfaces between gels. Most of the material migrates in one band which appears, by visual estimation, to contain about 90% of the total protein (Fig. 9). In favorable runs, this band appears to be resolved into two closely spaced subbands of equal intensity, but we have not yet excluded the possibility that this represents an artifact. The main band is identical in all runs,
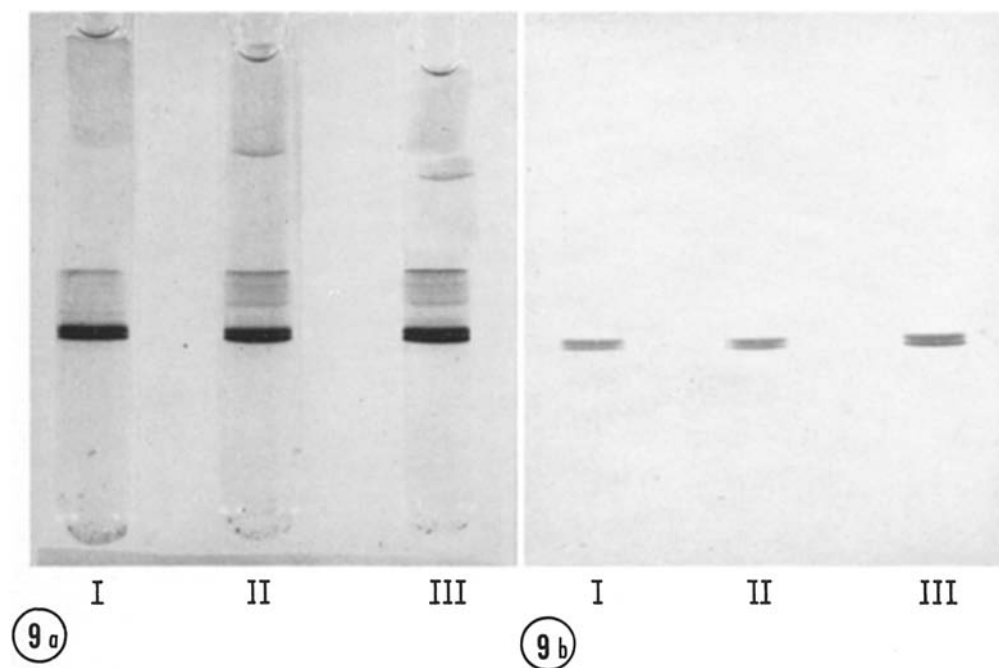


FIGURE 9 Disc electrophoresis in polyacryamide gels made with 8 M urea. All protein samples were reduced and alkylated (see text). Samples of outer-fiber protein from procedures 1, 2, and 3 were run simultaneously. *a*, printed darkly to show secondary bands; *b*, printed lightly to show doubleness of principal band.

regardless of the procedure employed to isolate the protein. About 10% of the outer-fiber protein appear as secondary bands moving behind the main one. There are about five or six of these, very faint, but sharp. The proportion of main band material to secondary bands seems to be fairly constant in preparations made by the same procedure. However, the positions of the secondary bands in protein from procedure 1 do not coincide with those from procedures 2 and 3. Essentially the same results are obtained when the protein had been reduced but not alkylated, or alkylated but not reduced.

The electrophoretic mobility of the major band of outer-fiber protein is identical with that of rabbit actin that has been reduced and alkylated (Fig. 10). The actin band, however, although broad, shows no indication of doubleness.

## Optical Rotatory Dispersion

With the assistance of Doctors Carolyn Cohen and Ray Stephens, we have made a preliminary measurement of the optical rotatory dispersion of outer-fiber protein. The data obtained from a sample of procedure 1 protein in 1 mM tris buffer gave a straight line when plotted according to the method of Moffitt and Yang (41). The slope of this line gave a $b_o$ of $-170$. Since 100% helix corresponds to a $b_o$ of $-630$, this indicates that the



FIGURE 11 Reaction of sulfhydryl groups of outer-fiber protein (procedure 1) with dithio-bis-nitrobenzoic acid. The time at which urea was added is indicated.
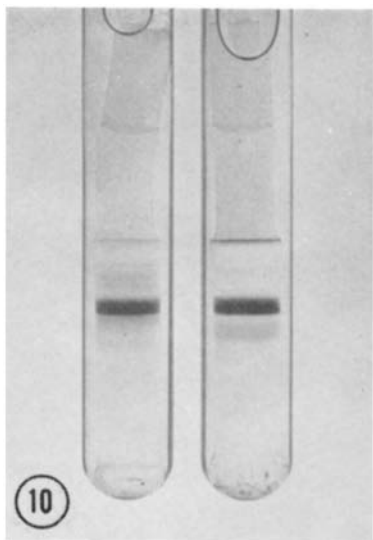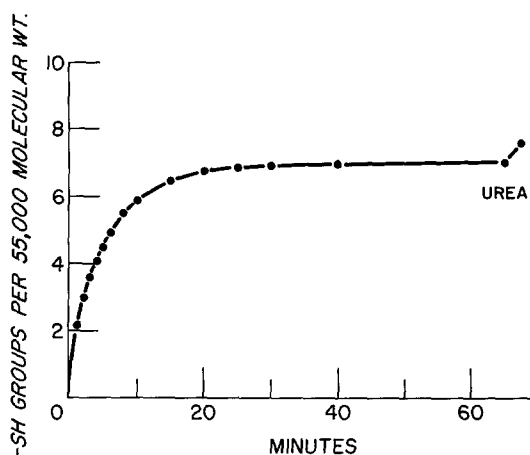


FIGURE 10 Actin from rabbit psoas muscle (right) was run simultaneously with outer-fiber protein from procedure 1 (left). Other details are the same as in Fig. 9.

outer-fiber protein contains approximately 28% $\alpha$-helix under these conditions.

## Amino-Acid Analysis

Protein that is freshly prepared by procedure 1 contains about seven sulfhydryl groups per mole of protein (55,000 g) that are capable of reacting with DTNB in the absence of a denaturing agent, plus an additional half group that reacts only in the presence of 8 M urea (Fig. 11). The total number of sulfhydryl groups decreases by 5–10% per day when the protein is stored, presumably as a result of atmospheric oxidation. Outer-fiber protein prepared by procedures 2 and 3 usually contains 5–6 moles of sulfhydryl per 55,000 g of protein; this lower number reflects the greater oxidation that occurs during the more lengthy preparation procedure. The presence of a reducing agent (1 mM mercaptoethanol) during preparation did little to prevent the loss of sulfhydryl groups.

The reaction of the protein with DTNB in the absence of urea reaches a plateau in about 30 min (Fig. 11), whereas mercaptoethanol of the same sulfhydryl molarity reacts completely within 1 min. This difference in reaction rate suggests that only two to three of the sulfhydryl groups on the outer-fiber protein are freely accessible to the reagent, and that the other four to five groups react only after secondary configurational changes occur.

The amino acid composition of protein from procedure 1 is given in Table II. Essentially
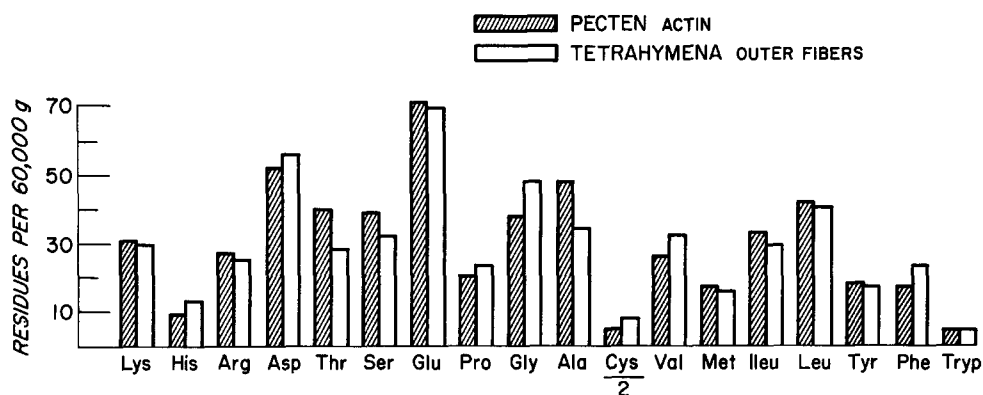
FIGURE 12 The amino acid composition of outer-fiber protein from cilia is compared with that of actin from *Pecten* muscle (5).

TABLE II

Amino Acid Composition of the Outer-Fiber Protein of Cilia*‡

| Amino acid§ | Residues per $10^5$ g of protein |
|---|---|
| Lysine | 51 |
| Histidine | 22 |
| Arginine | 41 |
| Aspartic acid | 94 |
| Threonine‖ | 46 |
| Serine‖ | 54 |
| Glutamic acid | 117 |
| Proline | 39 |
| Glycine | 80 |
| Alanine | 56 |
| Cysteine¶ | 13 |
| Valine | 53 |
| Methionine | 26 |
| Isoleucine | 49 |
| Leucine | 66 |
| Tyrosine | 29 |
| Phenylalanine | 39 |
| Tryptophan** | 7 |

* Ammonia was present, but was not determined with sufficient accuracy for a value to be reported.
‡ Protein samples were prepared by procedure 1.
§ Most values represent the average of three hydrolysis times on each of two samples.
‖ Extrapolated to zero hydrolysis time.
¶ Determined by reaction with DTNB.
** Determined from the spectrum in 0.1 N NaOH (2).

identical results were obtained with the protein from procedure 3. The composition shows fairly high amounts of the acidic amino acids, glutamic and aspartic, and of the hydrophobic amino

acids leucine, isoleucine, valine, and phenylalanine, but otherwise it is not particularly distinctive. The similarity of this amino acid composition to that of actin (Fig. 12) will be considered further in the Discussion.

## Partial Specific Volume

Determination with a pycnometer gave a value of 0.720 for the partial specific volume in tris buffer at 20°C. A value of 0.730 was calculated from the amino acid composition (7). The measured value of 0.720 was used in the molecular weight calculations, both for tris buffer and for guanidine hydrochloride solution.

## DISCUSSION

The evidence that the main protein component in our solutions derives from the outer fibers is fairly straightforward. In procedure 2, we obtain a preparation that appears by electron microscopy to contain purified bundles of outer fibers. About 95% of the protein in this preparation dissolves in 0.6 M KCl (12), and 90% of the protein in solution migrates in the principal electrophoretic band. Since the outer fibers were the only structures visible in the preparation, it is evident that the principal protein component must derive either from the outer fibers, or from a "matrix" that for some reason could not be seen in the electron micrographs. The latter possibility seems improbable, for it implies that the outer fibers account for only 5–10% of the total protein seen in electrophoresis. We conclude that the major protein in our solutions is the principal component of the outer fibers.

Supporting evidence is provided by the electron

microscopic observations of Grimstone and Klug (16), which show globular subunits 40 × 50 × 50 A as structural units of the outer fiber. Molecular weight calculation for a particle of this size gives a value of about 45,000, which is reasonably close to our measured value of 55,000 for the subunit of outer-fiber protein.

In previous work (29) we have reported the repolymerization of outer-fiber protein into fibrous tactoids composed in part of 40-A protofilaments, but we have not yet been able to induce the protein to form regular tubules resembling outer fibers. Whether this failure has resulted from the need for a preexisting "seed" to initiate the tubular form or from partial denaturation of the protein by our isolation technique, we are unable to say. Procedure 1 protein appears nearly monodisperse, and it is possible that the 6S dimer in these solutions represents a native form of the outer-fiber protein. However, in view of the general resemblance between the outer-fiber protein and actin, it must be noted that the 6S form of outer-fiber protein is more similar to the dimeric G-actin, produced upon inactivation with EDTA (23), than to native G-actin. It is also possible that the failure to polymerize is a result of the degradation of the nucleotide moiety of the outer-fiber protein that occurs during isolation (38).

The outer fibers in whole isolated cilia do not dissolve at low ionic strength, even after the ciliary membrane has been disrupted (procedures 2 and 3). However, the outer-fiber protein becomes readily extracted with 1 mM tris buffer after the cilia have been treated with acetone (procedure 1). The initial insolubility is possibly a result of some secondary links that stabilize the structure of the outer fibers until the acetone treatment renders them ineffective and permits the fiber protein to solubilize in dilute tris buffer. The absence of such links in the central fibers of the cilium would account for their ready solubility at low ionic strength. The chemical and structural nature of these postulated links remains obscure.

The principal protein of the outer fibers appears in electrophoresis as two closely moving subbands. This doubleness is possibly an artifact similar to that described by Smithies et al. (36) in the electrophoresis of reduced haptoglobins. If genuine, however, it would imply that the outer fibers contain equal amounts of two slightly dissimilar peptide chains. The difference in stability of the A and B subfibers in the doublet outer

fibers (3) also suggests a small difference in their composition.

We do not know whether the secondary bands found in electrophoresis of protein from procedures 2 and 3 represent minor components of the outer fibers or contamination absorbed onto the fibers from the matrix. The different secondary bands found in the protein obtained by procedure 1 presumably represent all the ciliary proteins which remain soluble at low ionic strength after acetone treatment. It has not been established whether the protein of the central fibers forms part of the main electrophoretic band or one of the secondary bands, in procedure 1.

In the only previous work on the outer-fiber protein, Watson and Hynes (43) have reported obtaining five major components on electrophoresis of the reduced and alkylated protein in starch gel at pH 8.9. The preparation of their protein was similar to our procedure 2, but the reduction and alkylation were performed without adding urea. The apparent discrepancy between their results and our finding of a single major component may be due to the different properties of starch and acrylamide gels, but we are more inclined to attribute the complex pattern obtained by Watson and Hynes to incomplete reduction of disulfide bonds that results from the omission of a dispersing agent from the reducing solution.

The sulfhydryl analyses of Watson and Hynes (43) indicate that their protein preparations were more highly oxidized than ours. For example, their assay for total cystine plus cysteine gave 5.5 moles —S— per mole of protein (55,000 g), but only about 1.5 moles of this was present as cysteine in the protein as prepared. This contrasts with our total assay of 7.5 moles, almost all present as cysteine in fresh preparations. We found our sulfhydryl titer to decrease by 10–15% per day when the protein was stored, and so we are inclined to believe that the low cysteine values of Watson and Hynes were an artifact, probably caused by autoxidation.

Our results suggest a model for the outer fiber in which stability is achieved by a combination of electrostatic and hydrophobic interactions between the protein subunits. This is consistent with the fact that fresh preparations of fibers break down almost completely to 55,000-molecular weight subunits in 5 M guanidine hydrochloride

(without reducing agent), and with the 60% swelling in volume of the fibers that occurs at low ionic strength (13). However, the reason for the increased solubility of the fibers after acetone treatment remains obscure, and the possible presence of a lipid cofactor cannot be excluded. We have no evidence for the presence of any disulfide bonds, either inter- or intramolecular, in the native fibers, but there are a fairly large number of exposed —SH groups which are susceptible to autoxidation during isolation and subsequent handling.

The outer fibers of cilia bear a close structural resemblance to cytoplasmic microtubules (28, 35), and it might be expected that they are composed of similar proteins. Borisy and Taylor (4) have identified a 6.0S component as the colchicine-binding unit in a variety of tissues rich in microtubules, and the properties of this component appear very similar to those of the outer-fiber protein. Shelanski and Taylor (34) have also extracted a 6.0S component from sperm flagella that has colchicine-binding capacities, and have identified it as coming from the central fibers. Sakai (31) and Kiefer et al. (21) have identified a 3.5S particle of molecular weight 68,000 as the principal constituent of microtubules in the mitotic apparatus. However, this particle can be split by reducing agents into two 2.5S particles of 34,000 molecular weight. The relationship of this mitotic apparatus protein to the outer-fiber protein of cilia is not yet clear, but the presence of an essential disulfide linkage in the former may explain why microtubules in the mitotic apparatus can be dispersed with reducing agents (25), while ciliary fibers can not. Kane (19) and Stephens (37) have described a different protein from the mitotic apparatus, which has a sedimentation coefficient of 22S and molecular weight of 880,000; this protein appears to be derived from a matrix associated with the microtubules rather than from the tubules themselves, and it has few properties in common with the outer-fiber protein.

It is now several years since attention was first drawn to the resemblance between the properties of outer-fiber protein and those of actin (12, 29). Actin is usually isolated from muscle, but its widespread distribution in cells of many types is made evident by its recent isolation from plasmodia of a myxomycete (18). The presence of an actin-like protein in sperm tails has been men-

tioned in preliminary reports by Ruby (30) and by Plowman and Nelson (27), although no evidence concerning the homogeneity and localization of these protein fractions has been published

The similarities and differences between outer fiber protein and actin may be summarized as follows. Both proteins are obtained by essentially the same isolation procedure involving extraction of an acetone powder at low ionic strength. The molecular weight of the outer-fiber protein in guanidine hydrochloride solution (55,000) is about the same as that of actin, probably 58,500 (22), although a lower value of 28,000 has also been reported (1). Outer-fiber protein and actin both migrate at the same velocity on electrophoresis in acrylamide gel at pH 8.9. The two proteins have closely similar amino acid compositions, with such differences as occur being of the same order as those between samples of actin from different species of animal (6). Both proteins contain approximately 1 mole of bound nucleotide per 55,000 g of protein, although outer-fiber protein contains a guanine nucleotide (38) while F-actin contains ADP (39). However, notwithstanding all these *chemical* similarities between the two proteins, it must be noted that there appears to be a marked *structural* difference, for the arrangement of subunits in the wall of an outer fiber (16) is quite different from that of the actin monomers in a secondary filament of muscle (17). Moreover, it has been reported that antibodies prepared against actin do not react with ciliary proteins (11), and also that actin does not bind colchicine (4). It is not yet possible to decide whether actin and outer-fiber protein should be regarded as the same protein, capable of fulfilling two roles in the cell, or as different proteins that are closely related in evolution. However, the latter possibility appears more probable. In any case, the two properties of actin which are particularly important in its known physiological function are its ability to form helical polymers as in the secondary filaments of muscle, and its ability to combine specifically with myosin and stimulate Mg-activated ATPase activity. Only if it is demonstrated that outer-fiber protein possesses these properties shall we be justified in referring to it by the name actin.

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