CHLAMYDOMONAS FLAGELLA

I. Isolation and Electrophoretic Analysis of Microtubules, Matrix, Membranes, and Mastigonemes

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ABSTRACT

Methods were developed for the isolation of *Chlamydomonas* flagella and for their fractionation into membrane, mastigoneme, "matrix," and axoneme components. Each component was studied by electron microscopy and acrylamide gel electrophoresis. Purified membranes retained their tripartite ultrastructure and were shown to contain one high molecular weight protein band on electrophoresis in sodium dodecyl sulfate (SDS)-urea gels. Isolated mastigonemes (hairlike structures which extend laterally from the flagellar membrane *in situ*) were of uniform size and were constructed of ellipsoidal subunits joined end to end. Electrophoretic analysis of mastigonemes indicated that they contained a single glycoprotein of $\sim$ 170,000 daltons The matrix fraction contained a number of proteins (particularly those of the amorphous material surrounding the microtubules), which became solubilized during membrane removal. Isolated axonemes retained the intact "9 + 2" microtubular structure and could be subfractionated by treatment with heat or detergent. Increasing concentrations of detergent solubilized axonemal microtubules in the following order: one of the two central tubules; the remaining central tubule and the outer wall of the B tubule; the remaining portions of the B tubule; the outer wall of the A tubule; the remainder of the A tubule with the exception of a ribbon of three protofilaments. These three protofilaments appeared to be the "partition" between the lumen of the A and B tubule. Electrophoretic analysis of isolated outer doublets of 9 + 2 flagella of wild-type cells and of "9 + 0" flagella of paralyzed mutants indicated that the outer doublets and central tubules were composed of two microtubule proteins (tubulins 1 and 2) Tubulins 1 and 2 were shown to have apparent molecular weights of 56,000 and 53,000 respectively

INTRODUCTION

The biflagellate, unicellular alga *Chlamydomonas* has been shown to have several unique advantages for studies on flagellar formation and function: it can be grown to high densities in defined medium (Sager and Granick, 1953; Gorman and Levine, 1965), its division can be synchronized by use of alternating light and dark periods (Bernstein, 1960; Kates and Jones, 1964), the flagella can be detached and isolated in large quantities, and the deflagellated cells will synchronously regenerate new flagella (Rosenbaum et al., 1969) There are, in addition, detailed ultrastructural analyses of the cell (Sager and Palade, 1957; Johnson and Porter, 1968) and its flagella (Ringo, 1967 a, 1967 b, Hopkins, 1970). Perhaps of even greater importance, mutants with defective flagella can be iso-

lated easily (Lewin, 1954, 1960) and genetically analyzed (Levine and Ebersold, 1960; Sager, 1964; Randall et al , 1964).

Studies on the development of *Chlamydomonas* flagella have been directed primarily toward the investigation of the in vivo synthesis, assembly, and turnover of flagellar proteins (Rosenbaum et al., 1969) By use of inhibitors of protein synthesis and microtubule assembly, it was demonstrated that flagellar development depended in part on preexisting flagellar precursors and in part on new protein synthesis, which was triggered by flagellar amputation. It was also found that assembly of the flagellum, particularly the microtubules, occurred at the distal tip of the elongating flagellum (Rosenbaum et al , 1969; Witman and Rosenbaum, unpublished results), and that little turnover of flagellar proteins took place in full-grown flagella (Gorovsky et al., 1970). Related studies by Coyne and Rosenbaum (1970) demonstrated that when one flagellum was amputated, the remaining flagellum was resorbed and that the resorbed proteins were reutilized in the subsequent formation of new flagella. Additional studies on the relation between flagellar precursor pools and the control of flagellar elongation are to be reported (Moulder, 1972; Moulder and Rosenbaum, in preparation). All of these findings, concerning various aspects of flagellar development in vivo, provide a firm foundation for studies on the formation of the individual components of the flagellum. However, before such studies could be initiated, it was first necessary to develop methods for flagellar fractionation.

In this report, methods for isolation and fractionation of wild-type and mutant *Chlamydomonas* flagella are described. Some of the techniques are new, while others are based on methods used by Gibbons (1963, 1965) for the isolation and fractionation of *Tetrahymena* cilia. By use of these procedures, *Chlamydomonas* flagella were fractionated into their membrane, mastigoneme, matrix, and microtubule components The outer doublets were then fractionated into A tubules, B tubule protein, and the common wall between the A and B tubules The protein composition of each of the major fractions was analyzed by urea and SDS-urea acrylamide gel electrophoresis The analysis of the outer doublets indicated that they contained two microtubule proteins. The arrangement of these two proteins in the walls of the outer doublet microtubules is treated in the following report (Witman et al , 1972), and their purification and

characterization is reported elsewhere (Olmsted et al , 1971; Carlson, Witman and Rosenbaum, in preparation).

MATERIALS AND METHODS

Cultures

Two strains of *Chlamydomonas* were used: 21-gr (wild-type) and pf-18 (paralyzed mutant). Cultures were grown in Medium I of Sager and Granick (1953) in 250-ml Erlenmeyer flasks, 2- and 5-liter diptheria toxin bottles (Pyrex 1295), and 8-liter solution bottles (Pyrex 1595). All cultures were incubated at room temperature (ca 25°C) and bubbled with air; cultures in 8-liter bottles were stirred magnetically. All of the experiments reported here were carried out on cells synchronized by a 14 hr light and 10 hr dark cycle (Bernstein, 1960; Kates and Jones, 1964). In some experiments with wild-type cells, sodium acetate (3 g/liter) was included in the medium to permit the cultures to reach much higher densities than they would on the Sager and Granick Medium I. When acetate was used, three times the normal concentration of phosphate buffer was included in the medium. Although the synchrony of division was still maintained in the presence of acetate, division was usually not completed until about 2 hr of the light cycle had occurred, whereas on minimal medium, division was completed during the dark cycle. Acetate was not used to culture the paralyzed mutant pf-18

Harvesting of Cells

Cells in 100 to 1000-ml cultures were harvested at room temperature by centrifugation in an IEC (International Equipment Company, Needham Heights, Mass.) PR-6 centrifuge with either 50-ml conical tubes and the No. 253 swinging bucket head at 550 g (1450 rpm) for 5 min, or 250-ml bottles and the No. 284 swinging bucket head at 1000 g (2200 rpm) for 6–8 min. Cells in larger cultures were harvested with a De Laval cream separator (De Laval Separator Co , Poughkeepsie, N. Y , Model No. 104, Cow to Can, with the cream outlet of the rotor plugged with solder) operated at half speed. The concentrated cells were resuspended in 10 mM Tris,[1,2] and washed once with the same buffer by centrifugation in 250-ml bottles as described above.

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[1] *Abbreviations used:* disodium EDTA, disodium ethylenediaminetetraacetate, ME, 2-mercaptoethanol; PTA, phosphotungstic acid, SDS, sodium dodecylsulfate, Tris, Tris (hydroxymethyl) aminomethane.

[2] All Tris buffered solutions used in these studies were adjusted with HCl to pH 7.8 at 23°C unless stated otherwise.

The flagella were detached and isolated from the concentrated cells by one of the following procedures.

Flagellar Detachment and Isolation

(1) SUCROSE-PH METHOD This procedure was a modification of the method originally described for the removal of flagella from *Euglena* (Rosenbaum and Child, 1967). The cells were harvested from 10 mM Tris and were resuspended in cold 10 mM Tris containing 5% sucrose All of the subsequent operations were performed at 2–4°C While stirring the suspension vigorously with a magnetic stirrer, the pH of the concentrated cell suspension was quickly lowered to 4 5 by addition of 0.5 N acetic acid The flagella became detached in about 30 sec The pH was then raised to 7 0 by addition of 0 5 N KOH or Tris base Flagellar detachment was independent of the volume of the suspension and of the cell concentration (up to 6 × 10⁸ cells/ml) 10 to 15-ml samples of the suspension of cells and flagella were then placed in 50-ml conical, polycarbonate tubes and underlayed with 15–20 ml of 25% sucrose, 10 mM Tris. Centrifugation at 3000 g (IEC PR-6, swinging bucket head No. 253, 3200 rpm) for 10 min sedimented the cell bodies through the 25% sucrose while the flagella remained in the 5% sucrose and at the interface between the two sucrose solutions. The interface and the 5% sucrose layer containing the flagella were removed, underlayed again with 25% sucrose, 10 mM Tris, and centrifuged as above to remove any remaining cell bodies. The cell-free interface and 5% sucrose layer were then removed and centrifuged at 31,000 g (Sorvall RC-2, SS-34 rotor, 16,000 rpm) for 20 min to sediment the flagella.

(2) STEEP + CALCIUM PROCEDURE The flagella were also isolated from *Chlamydomonas* by a modification of Gibbons' procedure for isolating cilia from *Tetrahymena* (Gibbons, 1965). The concentrated and washed cells were resuspended in an equal volume of 10 mM Tris and cooled to 10°C. As soon as the cells were chilled, four to five times the cell suspension volume of cold (1–2°C) Gibbons' solution (0.15 M sucrose; 15 mM Tris, 2.5 mM disodium ethylenediaminetetraacetate (disodium EDTA), 11% EtOH, 30 mM KCl this solution will be called "STEEP"[3] was added and the suspension stirred vigorously on a magnetic stirrer. Sufficient 1 M calcium chloride was then added to give a final concentration of 15 mM for wild-type (21-gr) cells and 18 mM for the paralyzed mutant (pf-18). Deflagellation was complete in about 2 min. The flagella were then separated from

[3] Warner (unpublished results) has found that a solution containing 10% ethanol, 5% sucrose, 30 mM KCl, 2 mM MgCl₂, 1 mM EGTA, and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) adjusted to pH 7 2–7.4 at 23°C can be substituted for the STEEP solution

the cell bodies by the centrifugation procedure described above for sucrose-pH flagella These flagella will be referred to as "STEEP + calcium" flagella It was important in the isolation of flagella by this procedure that no more than the specified amounts of calcium were added, otherwise the flagella, although intact, were resistant to further fractionation by some of the procedures used. The method was successful for pf-18 and 21-gr cells grown in minimal medium, but did not work consistently for cells grown in medium supplemented with acetate.

Electrophoresis

(1) UREA GELS · The proteins of whole flagella or fractions thereof were prepared for acrylamide gel electrophoresis by treatment with 8 M urea, reduction with 2-mercaptoethanol (ME) or dithiothreitol, and alkylation with iodoacetic acid, all by use of standard procedures (Hirs, 1967, Renaud et al., 1968) The proteins were separated by electrophoresis at 2–3. ma/gel in 7.5% polyacrylamide gels (Ornstein, 1964, Davis, 1964) containing 8 M urea. The gels were 6 mm in diameter and ca. 75 mm long. For determination of the relative amounts of various proteins, the gels were stained with fast green and quantitated as described by Gorovsky et al. (1970) Fast green has been shown to give the same color yield for different proteins and to stain flagellar proteins in amounts up to 150–200 µg/6 mm diameter gel with no appreciable deviation from linearity (Gorovsky et al, 1970). In the present study the amount of protein applied to each gel was between 20 and 60 µg. Urea acrylamide gels were usually run in duplicate so that one gel could be stained with Coomassie brilliant blue (Chrambach et al., 1967). This dye is more sensitive than fast green and was useful in detecting proteins present in very low concentrations

(2) SDS-UREA GELS Proteins were also analyzed on SDS-urea acrylamide gels by a modification of the method described by Laemmli (1970). Proteins were dissolved in a solution containing 8 M urea, 2% SDS, 5% ME, and 0.0625 M Tris (pH 6 8 at 23°C) by heating for 3 min in boiling water. The proteins were then separated by electrophoresis at 2 ma/gel in a discontinuous gel system identical to that used for urea gels (see above) except that all gel solutions and buffers contained 0.1% SDS Gels were fixed in 50% trichloroacetic acid and stained with Coomassie brilliant blue as described by Laemmli (1970)

Determination of Protein Concentration

The concentration of protein in flagellar fractions was determined by the method of Lowry et al. (1951) using serum albumin standards

Electron Microscopy

(1) THIN SECTIONS: All flagellar fractions were placed in 13.5-ml polycarbonate (Oak Ridge) tubes and centrifuged at 133,000 g (Spinco L2-65B, 50-Ti rotor, 45,000 rpm) for 45 min. The supernatants were removed and the pellets fixed in situ for 60 min with 2.5% glutaraldehyde in 10 mM potassium phosphate buffer, pH 7.0, (usually at 0°C), and post-fixed for 60 min in 1.0% osmium tetroxide at 0°C in the same buffer. The pellets were dehydrated in situ in 50, 70, 95, and 100% alcohols, and then gently teased from the tube wall with a needle or small spatula. The intact pellets were transferred to a glass vial for further dehydration in propylene oxide, followed by infiltration and flat embedding in either Maraglas (Erlandson, 1964), Epon, or Araldite (Luft, 1961). The pellets were sectioned with a Porter-Blum MT-2 microtome. Because flagella are elongate, they usually were oriented with their longitudinal axes in the plane of the pellet; therefore, sectioning at 90° to the plane of the pellet resulted in many flagellar cross-sections in each thin section (see Fig. 10). This technique greatly facilitated the quantitative analysis of flagellar microtubules in the various fractions as described in the following report (Witman et al., 1972). The sections were mounted on uncoated 400-mesh, or Formvar-coated 200-mesh grids then stained with uranyl acetate and lead citrate (Reynolds, 1963; Venable and Coggeshall, 1965) and observed with a Philips 200 electron microscope.

(2) NEGATIVE STAINS: A drop of the flagellar fraction was placed on a Formvar- and carbon-coated 400-mesh grid. After a few minutes, most of the drop was removed with a pipette. A drop of 4% phosphotungstic acid in 0.4% sucrose (pH 7.0) was placed on the grid and immediately drawn off with a piece of filter paper. The grid was then examined with a Philips 200 or 300 electron microscope.

RESULTS

(I) Appearance of Isolated Flagella

The integrity of the isolated flagella of Chlamydomonas differed depending on whether they were detached by the sucrose-pH or the STEEP + calcium procedure. Their appearance as judged by light microscopy with phase and Nomarski optics and by electron microscopy of thin-sectioned material is described below.

(A) STEEP + CALCIUM FLAGELLA

The flagella isolated by this procedure appeared straight and intact when observed by phase or Nomarski microscopy (Fig. 1). There was no ob-

vious membrane fraying even after the preparation had remained in STEEP + calcium for several hours. Electron microscopy revealed that almost all of these flagella had intact membranes and axonemes and still retained their flagellar sheaths and mastigonemes (Fig 3). The matrix in which the axonemes were embedded appeared to be much denser than in flagella in situ The condensing of the matrix was apparently due to membrane shrinkage during flagellar isolation, since the isolated flagella had considerably smaller diameters than flagella in situ. The membrane shrinkage also squeezed the outer doublets closer together, so that the diameters of the axonemes were smaller in isolated flagella with intact membranes (Fig 3) than in flagella with disrupted membranes (see sucrose-pH flagella in Fig. 4) or than in flagella in situ (see Figs. 43 and 48 in Ringo, 1967 a, and Fig. 7 in Ringo, 1967 b) In many cases, one or more of the outer doublets were displaced toward the center of the axoneme (Fig. 3, arrows).

(B) SUCROSE-PH FLAGELLA

Observations of sucrose-pH flagella by phase or Nomarski optics indicated that most of the flagella were straight but, in contrast to the STEEP + calcium flagella described above, a variable portion appeared to have ragged membranes, to be thinner, and to be stuck to the glass slide or cover slip.

When sucrose-pH flagella were harvested and resuspended in medium lacking sucrose (e.g. 10 mM Tris, pH 7.0), the flagella with frayed membranes remained straight, while those with intact membranes swelled (Fig. 2) so that they appeared round or discoid. Consequently, suspending a sample of sucrose-pH flagella in medium lacking sucrose and observing the percentage of flagella which were swollen could be used as a rapid method for assaying the number of flagella having intact membranes.

Electron microscopy indicated that some sucrose-pH flagella had intact membranes and were very dense as described above for the STEEP + calcium flagella, while others had disrupted membranes (Fig. 4). The flagella with disrupted membranes had lost some of their matrix, and often one of the two central tubules and the outer portion of some of the B tubules had been dissolved. Although these "partially-intact" flagella could not be used as starting material for isolating intact 9 + 2
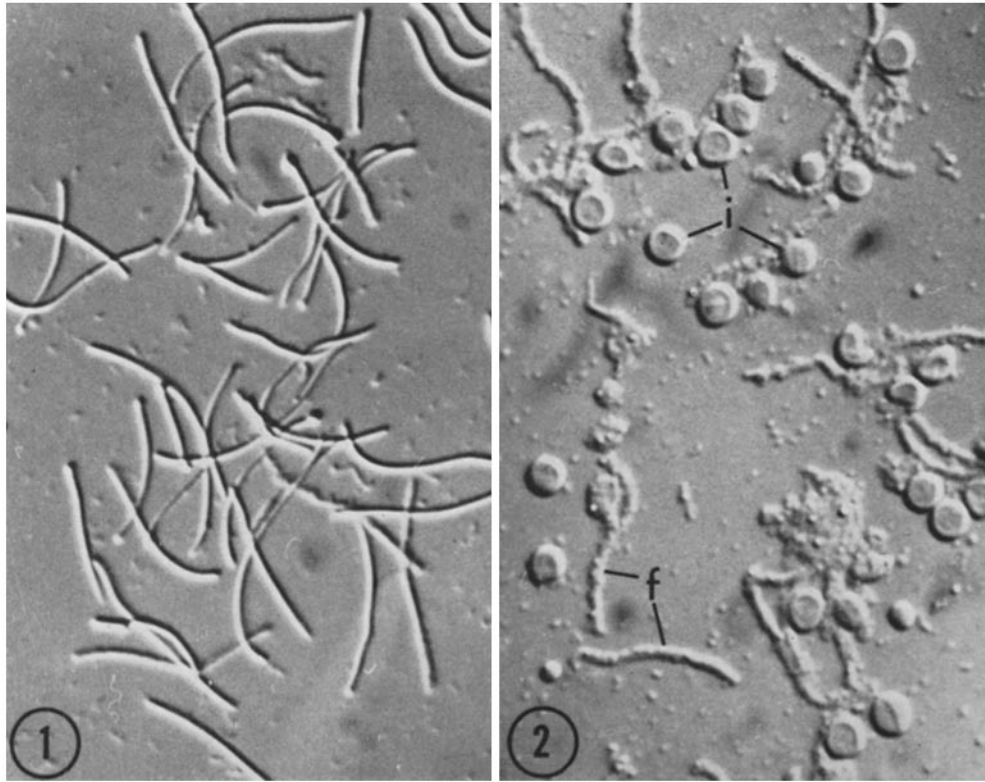
Figures 1 and 2 Nomarski micrographs of isolated flagella. × 2000 (Approximate). Fig. 1, Flagella isolated by the STEEP + calcium procedure. The flagella appear straight and intact. Fig. 2, Flagella isolated by the sucrose-pH procedure and resuspended in 10 mᴍ Tris, pH 7.0. The flagella with frayed membranes remain straight (*f*); those with intact membranes swell (*i*).

axonemes, they were useful for isolating outer doublet microtubules (see below).

In general, although the flagella isolated by the sucrose-pH procedure were not as intact as those isolated by the STEEP + calcium method, the sucrose-pH procedure was easier, quicker, and more manageable, especially when large volumes of cells were used. It was also milder in that it did not require the use of ethanol, EDTA, or calcium.

Various procedures were tried for removing the membranes from the intact STEEP + calcium flagella without simultaneously dissolving parts of the 9 + 2 microtubular axonemes. Among these were treatment with detergents, mechanical agitation, osmotic lysis, and dialysis against low ionic strength solutions at different pHs. Three methods proved satisfactory; in two of these methods the detached membranes remained morphologically intact, while in the third method the membranes

were dissolved These three procedures are described below·

(II) *Detachment of Flagellar Membranes From Axonemes*

(A) Steep + Calcium Flagella

(1) steep minus calcium rinse: Flagella isolated by the STEEP + calcium procedure were resuspended in STEEP *without* calcium, using 3 ml of this solution per original 7 5 ml of packed cells. The resuspension was carried out in the cold (2–4°C) and at no time was the temperature permitted to go over 4°C The suspension was vigorously pulled up and down through a Pasteur pipette until most of the "silkiness" characterizing preparations of intact flagella had disappeared and the turbid solution had cleared somewhat. This

usually took 5–10 min. Phase or Nomarski micro-scope observations (1000 ✕) showed that the demembranated flagella were straight and slightly less dense than the intact flagella, and were stuck to the slide or cover slip, in contrast to the intact flagella which moved freely in the solution The detached membranes were visible as very fine particles.

(2) NONIONIC DETERGENT: When STEEP + calcium flagella were treated with nonionic detergents such as Triton X-100 or Nonidet P-40 (Shell Chemicals, London SE 1, England), the membranes were not only efficiently removed, but almost entirely solubilized, leaving intact axonemes. To carry out this procedure, STEEP + calcium flagella from 4 liters of culture were suspended in 1–2 ml of cold STEEP minus calcium. An equal volume of 1% Nonidet P-40 made up in STEEP minus calcium was added to give a final detergent concentration of 0 5%. The suspension was agitated with a Pasteur pipette until phase microscope observation indicated that most of the flagella had been demembranated. The suspension was then layered over 3.0 ml of 40% sucrose (in STEEP), and centrifuged at 16,000 g (Sorvall HB-4 swinging bucket rotor, 10,000 rpm) for 1 hr. The resulting supernatant contained detached mastigonemes (see below) and solubilized membrane proteins. The pellet contained intact axonemes.

(3) IONIC DETERGENTS: The membranes could also be removed from STEEP + calcium flagella by treatment with 0 1% Sarkosyl[4] in 10 mM Tris for 15–30 min at 4°C. Although this was a very effective method for removing the membranes without solubilizing them, the use of ionic detergents like Sarkosyl resulted in somewhat more microtubule breakdown than either of the two procedures described above.

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[4] Sarkosyl NL-97 (sodium lauroyl sarcosinate) was obtained from Geigy Chemical Corp., Ardsley, N. Y.

(B) SUCROSE-pH FLAGELLA

Two methods were used to detach membranes from axonemes of sucrose-pH flagella:

(1) IONIC DETERGENT: The flagella were treated at 4°C with 0.1% Sarkosyl in 10 mM Tris until phase or Nomarski microscope observations indicated that most of the membranes were detached from the axonemes (about 15–20 min) In contrast to the STEEP + calcium axonemes, the demembranated sucrose-pH axonemes were splayed out into fine fibrils at one end.

(2) LOW IONIC STRENGTH DIALYSIS: The membranes could also be removed from sucrose-pH flagella by resuspending the flagella in TEM solution (0.1 mM EDTA, 0 01% ME, 1 mM Tris, pH 8 0 at 23°C) and dialyzing them against 500 volumes of this solution at 4°C After 12–14 hr of dialysis the membranes had become detached from the axonemes, which were splayed out into fine fibrils along most of their lengths. As described below, these fibrils consisted primarily of separated outer doublets

(III) Separation of Flagellar Membranes from Axonemes

After detaching the membranes from the axonemes of STEEP + calcium or sucrose-pH flagella by any of the above methods (except the method utilizing nonionic detergent, where the membranes were solubilized) the membranes could be separated from the axonemes by either of the following procedures

(A) DIFFERENTIAL CENTRIFUGATION

The suspension of membranes and axonemes was layered over an equal volume of 40% sucrose (in STEEP for STEEP + calcium flagella or in 10 mM Tris for sucrose-pH flagella) in 12-ml round-bottomed tubes and centrifuged at 16,000 g

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FIGURE 3 Electron micrograph of flagella isolated by the STEEP + calcium procedure. The nine outer doublet microtubules and two central microtubules of the axoneme are intact and embedded in a densely-staining matrix. The flagellar membranes are mostly intact and appear to have contracted around the axoneme, decreasing the diameter of the flagellum and condensing the matrix. In some cases, one of the outer doublets has been forced towards the center of the axoneme (arrows). ✕ 63,000. Inset: Isolated flagella retain the flagellar sheath (fs). In outer doublets of many flagella, small beaklike projections (arrows) extend from the wall of the B tubule into the lumen of that tubule; these projections almost always occur in one outer doublet on one side of the central tubules and in two outer doublets on the opposite side. ✕ 118,000.
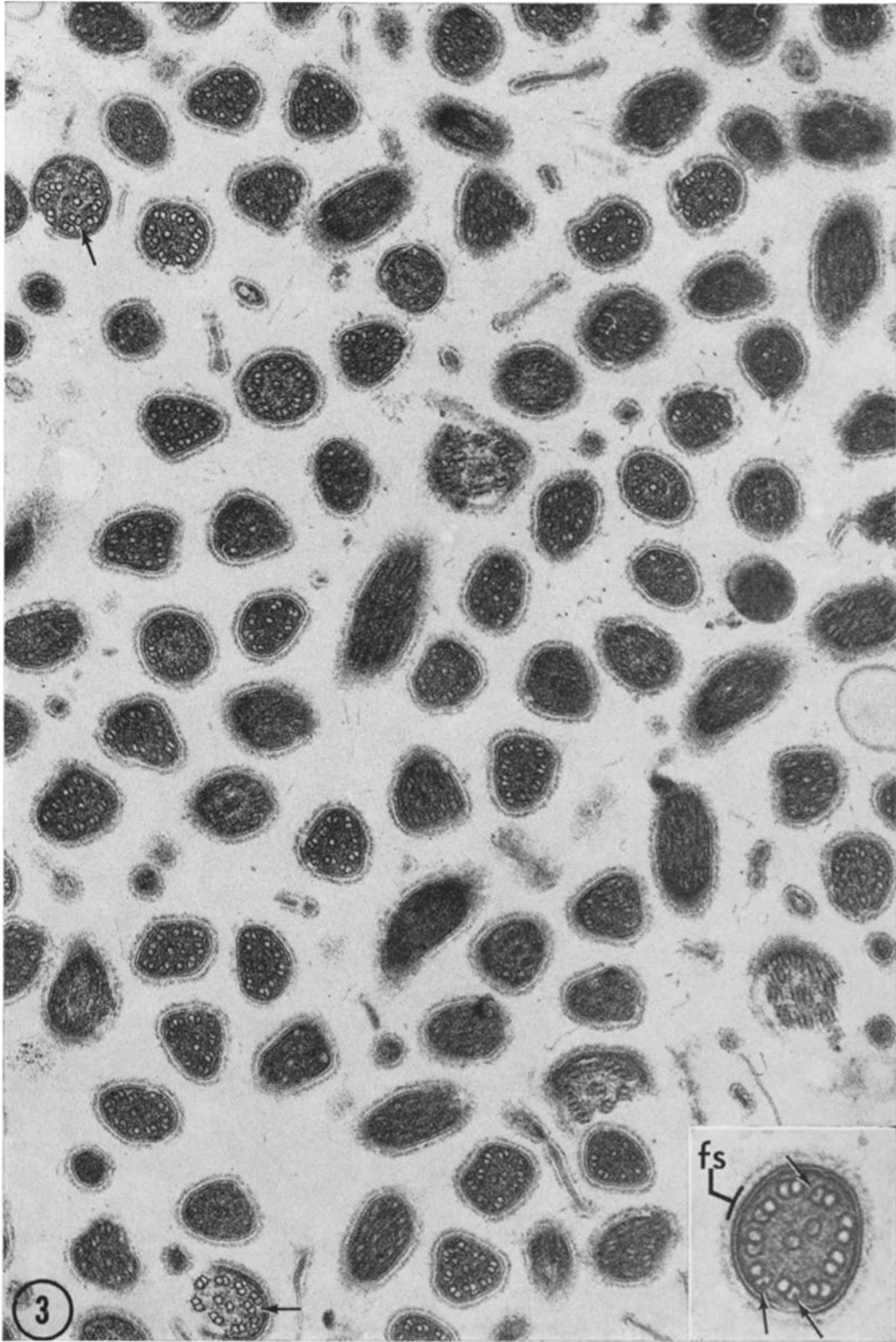
fs

FIGURE 4   Electron micrograph of flagella isolated by the sucrose-pH procedure. Some flagella remain intact, but in others the membranes are detached from the axonemes, and the densely-staining matrix is gone; in such flagella one of the two central microtubules and outer portions of the B tubule of the outer doublets are often solubilized. × 55,000.

(Sorvall HB-4 swinging bucket rotor, 10,000 rpm) for 60 min Most of the membranes remained at the interface between the original suspension and the 40% sucrose, while the axonemes were sedimented to the bottom of the tube. The interface was removed and centrifuged at 105,000 g (Spinco 50-Ti rotor, 40,000 rpm) for 60 min to obtain the membranes The membranes isolated by this procedure had the typical tripartite ultrastructure and appeared to be nearly free of microtubular components when judged by both electron microscope (Fig. 5) and electrophoretic analyses (see Sec. VIII B 3). For most of the work described, this differential centrifugation method was used

(B) Isopycnic Centrifugation

Alternatively, the membranes could be isolated by equilibrium centrifugation in sucrose Samples of the suspension of membranes and axonemes

were layered over linear 35–60% sucrose gradients in 10 mM Tris and centrifuged at 148,000 g (Spinco SW-41 rotor, 35,000 rpm) for 10 hr. The membranes banded isopycnically at about 45% sucrose while the axonemes were sedimented to the bottom The membrane band as it appeared in the centrifuge tube is shown in the inset of Fig 5. Although this procedure was more time-consuming than the separation by differential centrifugation described above, the resulting membrane fractions were cleaner when judged by both electron microscope and electrophoretic criteria.

(IV) Matrix

Various flagellar proteins, primarily those contained in the densely staining matrix, were released into solution upon detachment of the membrane from the axoneme. These matrix proteins remained in the supernatant after separation of
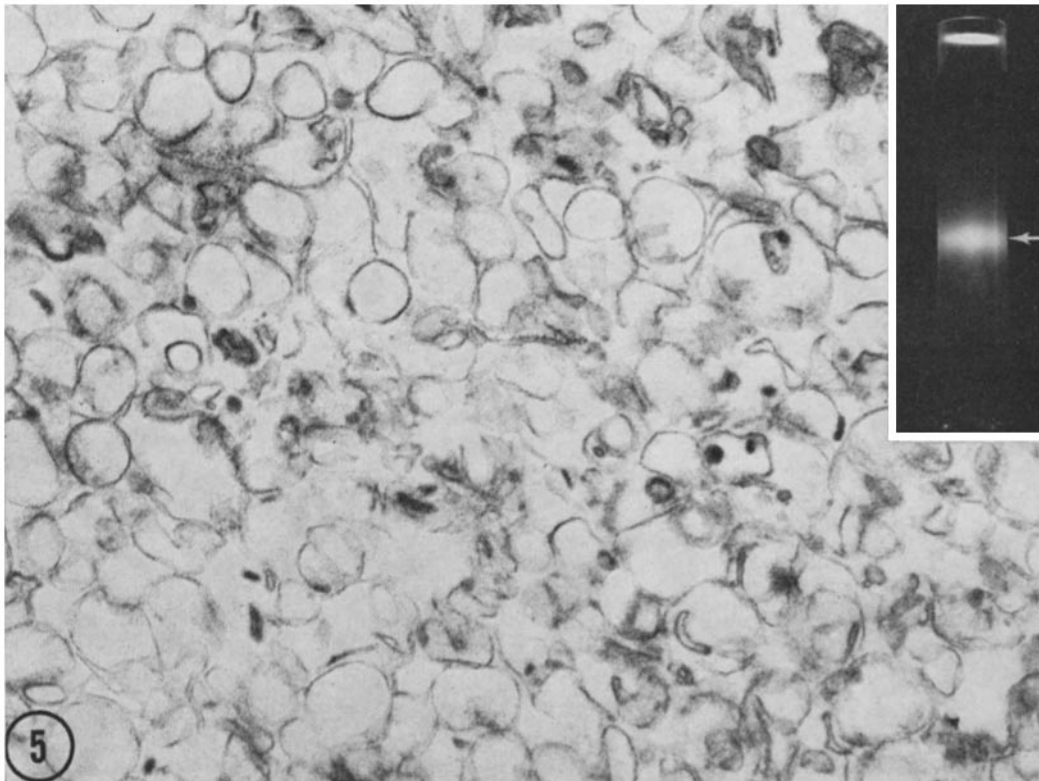

FIGURE 5 Electron micrograph of flagellar membranes which have been separated from axonemes by the differential centrifugation procedure. No axonemal components are apparent. × 42,000. *Inset:* Membranes which were separated from axonemes by isopycnic centrifugation formed a band (arrow) at about 45% sucrose.

the membranes from the axonemes by either of the above methods. Because the different procedures for membrane detachment solubilized different flagellar structures (see Sec VI A), the matrix fractions contained varying numbers and types of proteins For example, when membranes were detached from sucrose-pH flagella by the TEM dialysis procedure, more than 20 different flagellar proteins were released into solution (see Sec VII A 2 and Fig. 21), in contrast, when membranes were detached from STEEP + calcium flagella by the STEEP minus calcium rinse procedure, only a few proteins were solubilized (see Sec VII A 2 and Fig 23).

The matrix fractions were prepared for electrophoresis after removal of unsolubilized portions of the flagella by centrifugation at 105,000 g (Spinco 50-Ti rotor, 40,000 rpm) for 45 min. Only the top two thirds of the supernatant from this centrifugation were collected to avoid contaminating the matrix fraction with the pelleted material

(V) *Isolation of Mastigonemes*

Fine, threadlike projections called mastigonemes, flimmer, or flagellar hairs extend laterally from the membranes of *Chlamydomonas* flagella (Fig. 6, and see Fig. 42 in Ringo, 1967 a) The mastigonemes remained attached to the flagella during flagellar isolation by either the sucrose-pH or STEEP + calcium methods. They could then be detached from the isolated flagella and purified by a modification of the procedure designed by Bouck (1971) for isolating mastigonemes from *Ochromonas* flagella

Isolated *Chlamydomonas* flagella were suspended in 0.7% Sarkosyl in 10 mM Tris at 4°C After 6–8 hr the particulate material was sedimented by centrifugation at 105,000 g (Spinco 50-Ti rotor, 40,000 rpm) for 45 min The pellet was then resuspended in 3–5 ml of 2.80 M CsCl in 10 mM Tris This suspension was centrifuged at 124,000 g (Spinco SW 50L, 39,000 rpm) for 24 hr The mastigonemes formed a sharp white band in the center of the gradient; this band was removed with a Pasteur pipette or was collected by puncturing the bottom of the tube The CsCl was removed by dialyzing the mastigoneme fraction against 10 mM Tris, or by diluting the fraction with an equal volume of tris buffer and then sedimenting the mastigonemes by centrifugation at 105,000 g (Spinco 50 Ti rotor, 40,000 rpm) for 45
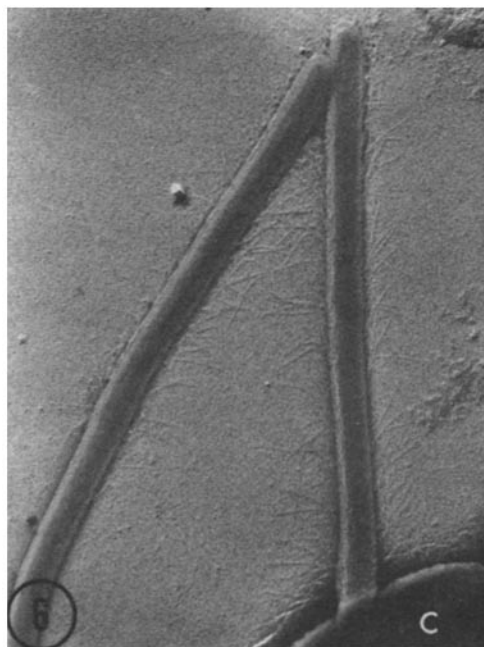

FIGURE 6 Electron micrograph of whole flagella *in situ* showing lateral attachment of mastigonemes. *c*, cell body. Whole cells were fixed in OsO₄ vapor and shadowed with 80% platinum —20% palladium. × 24,600.

min. Preparations of mastigonemes obtained in this manner were free of membrane or axoneme contaminants as determined by electron microscope (Fig 7) and electrophoretic criteria (see Secs VIII A 5 and VIII B 4).

The mastigonemes could also be isolated by a more rapid procedure utilizing Nonidet P-40 As noted above (Section II A 2), treatment of flagella with this nonionic detergent solubilized most of the membranes and released the mastigonemes into solution When the suspension of solubilized membrane proteins, mastigonemes, and axonemes was layered over 40% sucrose and centrifuged at 16,000 g for 1 hr, the axonemes were sedimented to the bottom of the tube, while most of the mastigonemes were retained at the interface between the two layers. The interface and upper layer could then be collected, pooled, and centrifuged at 105,000 g (Spinco 50-Ti rotor, 40,000 rpm) for 45 min to yield a pellet of mastigonemes

When purified *Chlamydomonas* mastigonemes were negatively stained with phosphotungstic acid (PTA) and observed by electron microscopy, they
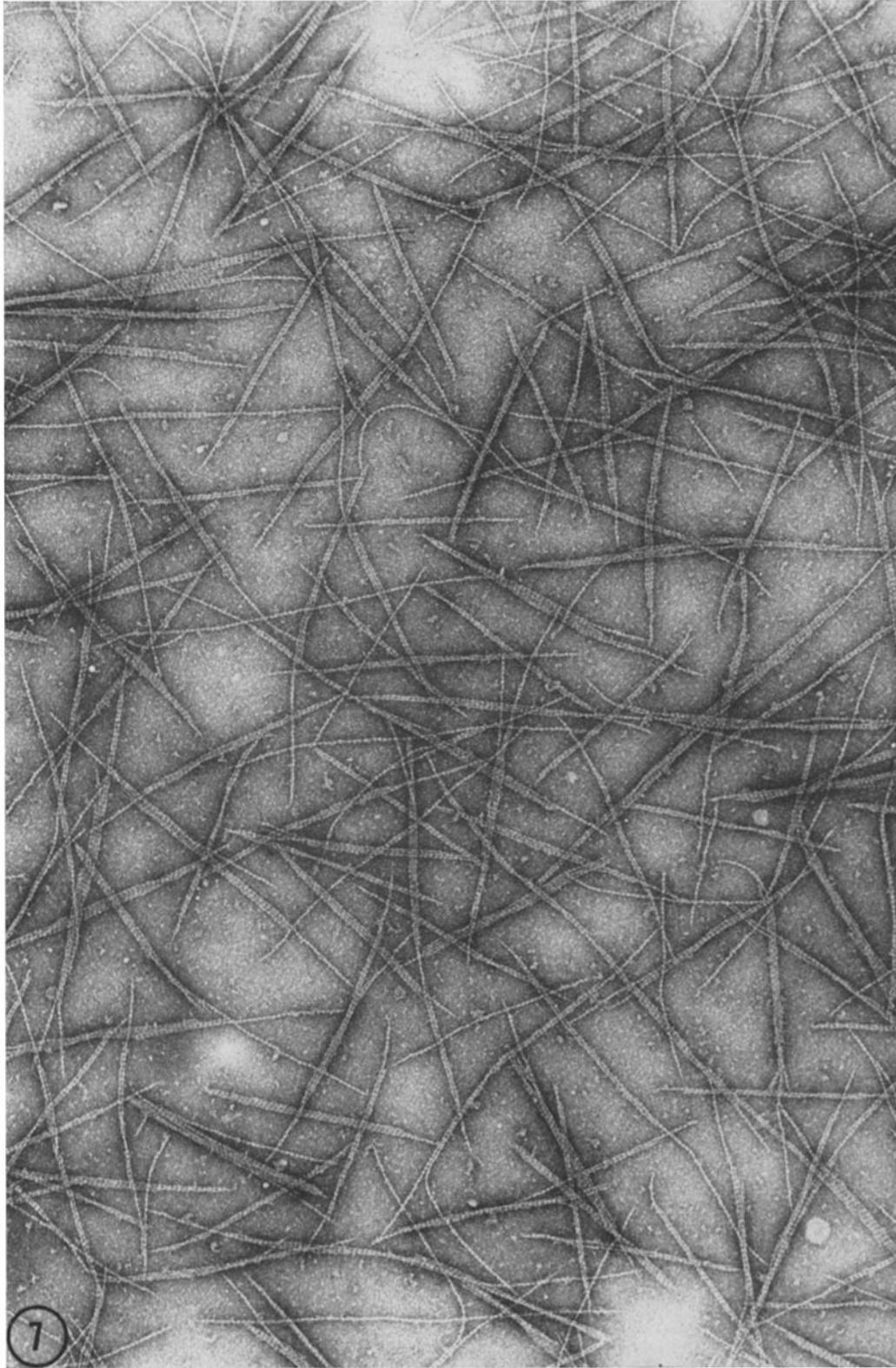
FIGURE 7 Electron micrograph of isolated mastigonemes negatively stained with PTA. Some of the mastigonemes have associated side by side to form small bundles. Each mastigoneme is about 0 9 μ long, × 37,600.

appeared as filaments ca. 0 9 μ long composed of a single row of subunits (Fig. 8) The subunits were each approximately 160 A wide by 200 A long, and were aligned end to end along their long axes. When the mastigonemes were concentrated, they tended to associate laterally into bundles exhibiting distinct cross-striations with a periodicity of about 200 A (Fig 9).

(VI) *Fractionation of Flagellar Microtubules*

As described above, the flagellar membranes could be detached from isolated flagella by various procedures and the intact or solubilized membrane material separated from the axonemes by differential or isopycnic centrifugation. In both cases, the axonemes were sedimented to the bottom of the tube.

In order to establish the sequence of solubilization of the outer doublet microtubules of the axonemes in the results which follow, it was necessary to be able to distinguish the A tubule from the B tubule at various stages of the solubilization procedure and, moreover, to be able to tell which were the outer and inner portions of each tubule. This could be done because in cross-sections of intact outer doublets the A tubule is easily recognizable by its round shape, while the B tubule is in the form of a slightly-flattened "C" attached to the A tubule. In addition, the outer doublets are on an angle with the B tubule canted outward and, as described below, certain of the B tubules have a spike projecting into their lumens (Fig. 3, insert). The inner and outer portions of the A and B tubules could be differentiated even after solubilization had progressed to the point where only parts of the A tubule remained because the normal circular arrangement of the partially-solubilized outer doublets tended to be maintained This maintenance of the circular arrangement of the outer doublets during their solubilization was particularly evident when the axonemes were isolated by the STEEP + calcium procedure, (cf Fig. 15) but less so when the outer doublets were isolated by the sucrose-pH method where they tended to lose their relationship with one another soon after the B tubules became solubilized (cf Fig. 11).

(A) Appearance of Isolated Axonemes

Axonemes obtained by the STEEP minus calcium rinse procedure (Sec II A 1) are shown in Fig. 10 Some membrane was still present. Axo-

nemes obtained by rinsing the flagella in STEEP containing Nonidet P-40 (Sec II A 2) were similar in appearance except that little or no membrane was evident. The 9 + 2 microtubular structure was completely intact in most axonemes, although occasionally portions of one of the central tubules and the outer portions of some of the B tubules were solubilized. These axonemes retained the radial links, the arms on the A tubule of the outer doublets (dynein), and the peripheral links between the A and B tubules of adjacent outer doublets (see insets, Fig. 10). Because of the high degree of integrity of the axonemes obtained by these procedures, they provided favorable starting material for the stepwise fractionation of the axonemal microtubules.

Axonemes obtained by treatment of isolated STEEP + calcium or sucrose-pH flagella with 0 1% Sarkosyl appeared similar to those described above except that in a greater proportion of the axonemes one of the central tubules and the outer portions of some of the B tubules were solubilized.

Dialysis of sucrose-pH flagella against TEM (Sec. II B 2) disrupted most of the axonemes; the resulting preparations contained primarily separate outer doublets or outer doublets held together in loose groups of two to nine (Fig. 11). The central tubules, the arms on the A tubule of the outer doublets, the radial links, and most of the peripheral links were solubilized. In many of the outer doublets, the outer portion of the B tubule was also solubilized

(B) Fractionation of Axonemes by Treatment with Sarkosyl

The microtubules of isolated axonemes could be sequentially solubilized by treatment with increasing concentrations of the detergent Sarkosyl. In the following procedures, the Sarkosyl was dissolved in 10 mM Tris, and all treatments with the detergent were carried out at 2–4°C for approximately 1 hr, unless stated otherwise.

(1) central tubules: Treatment of intact axonemes, such as those shown in Fig 10, with 0.1–0.15% Sarkosyl resulted in the removal of one of the two central tubules; in some experiments, preparations were obtained which contained primarily "9 + 1" axonemes (Fig. 12). In such preparations, there were a few axonemes which still contained both central tubules and a few with both removed; in some axonemes the
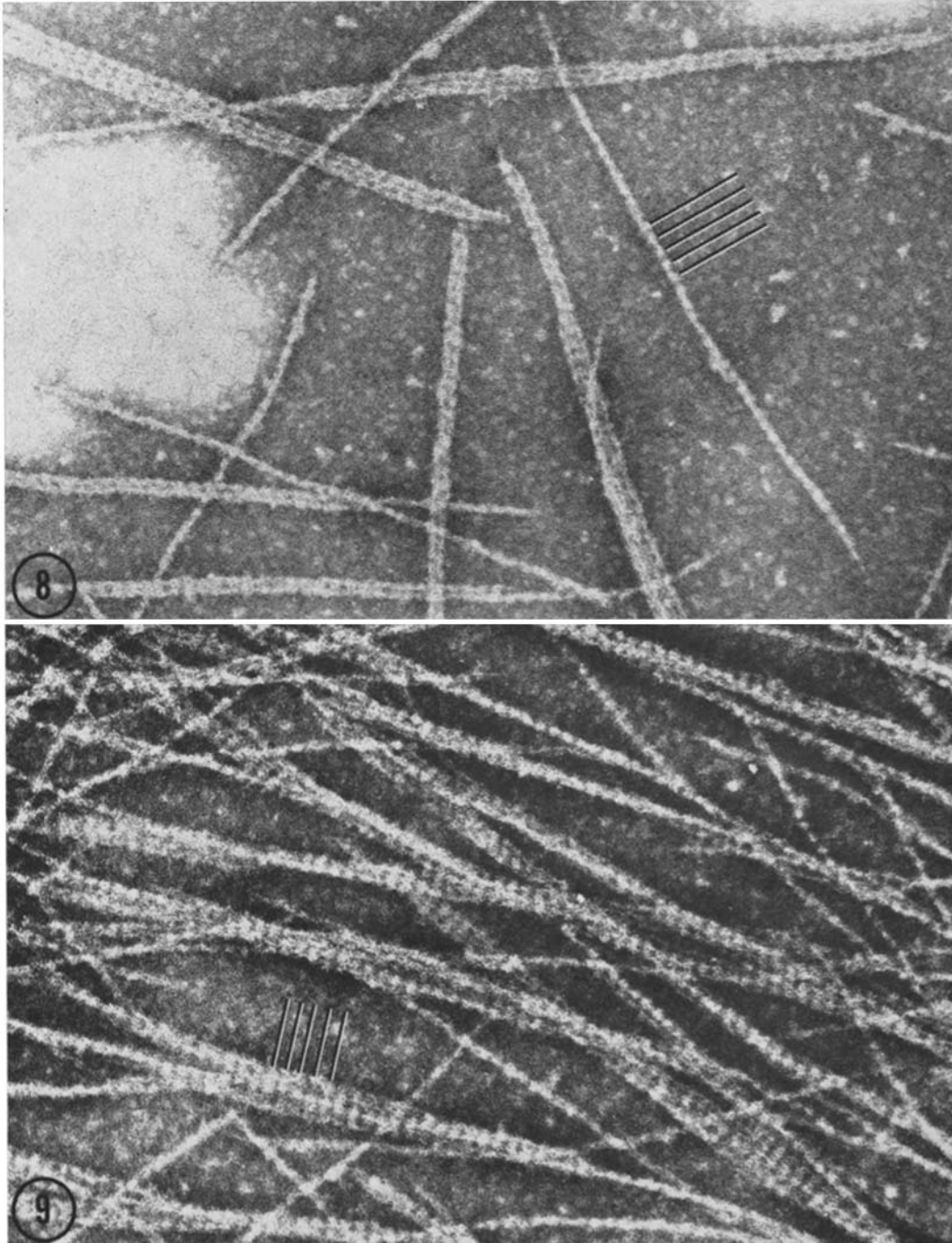
Figures 8 and 9 Isolated mastigonemes negatively stained with PTA. × 101,000. Fig. 8, Each mastigoneme appears to be composed of a single row of subunits (bars) having a center-to-center spacing of ca. 200 A. Fig. 9, In higher concentrations the mastigonemes become aligned laterally into bundles exhibiting distinct cross striations having a periodicity of ca. 200 A (bars).
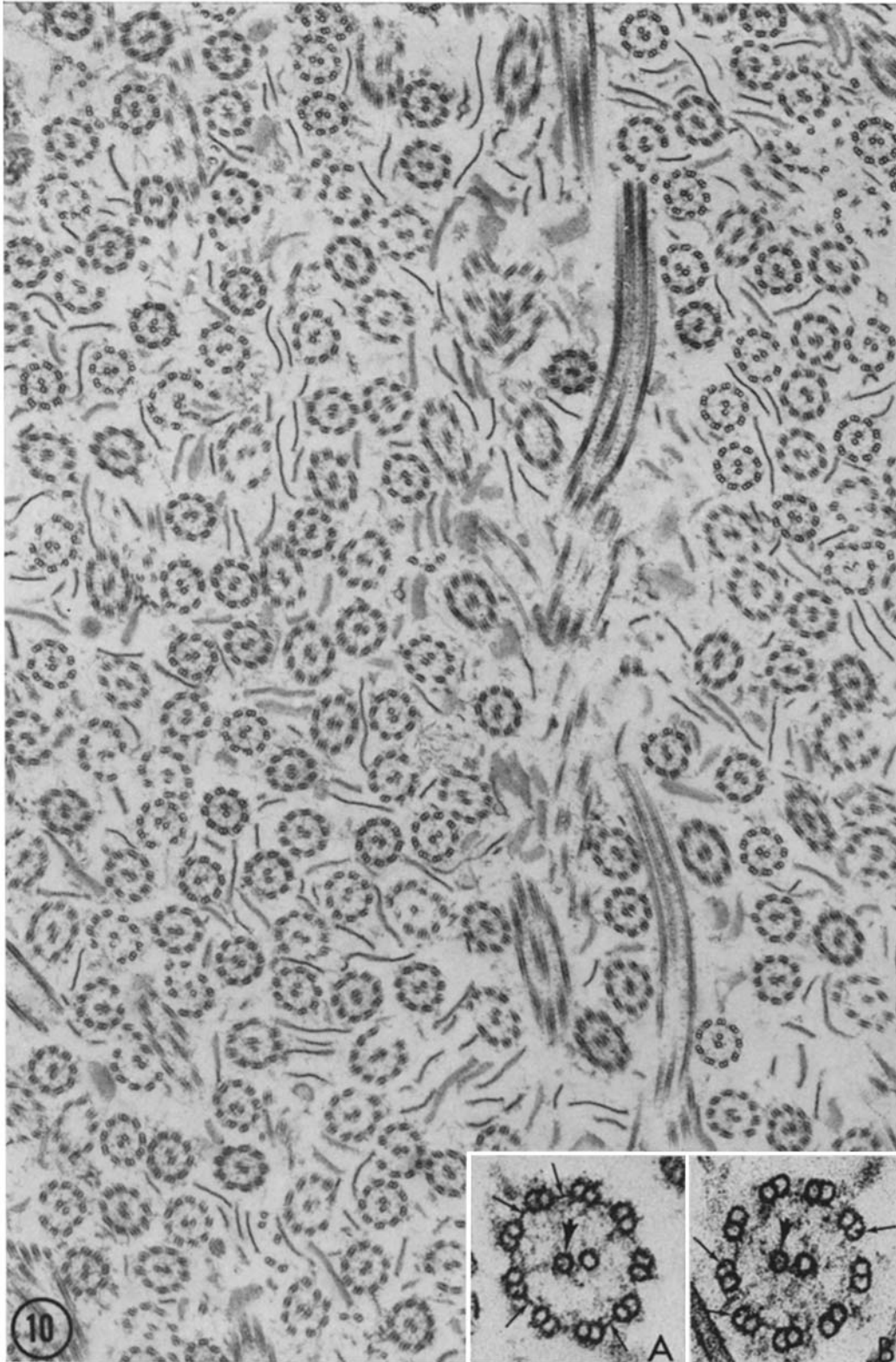
FIGURE 10   Electron micrograph of axonemes isolated by the STEEP minus calcium rinse procedure. In most cases the nine outer doublet microtubules and two central tubules are present and intact. × 33,000. *Insets:* In some isolated axonemes the lumen of one of the central tubules (large arrowheads) contains electron-opaque material. × 114,000. *A,* In some axonemes, thin rodlike links (arrows) connect the A tubule of one outer doublet to the B tubule of an adjacent outer doublet. *B,* The B tubules of many isolated axonemes retain their intratubular projections (arrows).
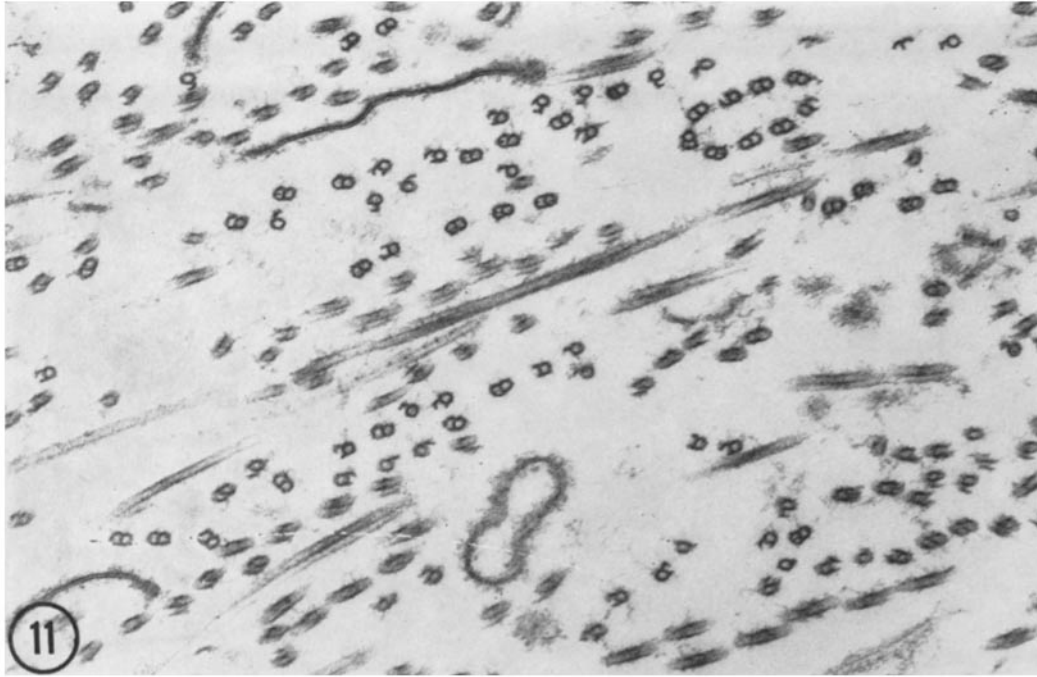
FIGURE 11   Outer doublets isolated by the TEM dialysis procedure. The central tubules, matrix, and most of the intermicrotubular connecting structures such as the radial and peripheral links have been solubilized. In many outer doublets a portion of the B tubule has also been solubilized. × 74,000.

outer portions of the B tubule of the outer doublets were also solubilized The occurrence of axonemes with only one central tubule in these preparations was much more frequent than would be expected from random solubilization of the two central tubules This observation clearly indicated that *the two central tubules differed in their solubility properties and that one was preferentially solubilized.* It has been shown that one of the central tubules of *Chlamydomonas* has two longitudinal rows of short "arms" and that the other central tubule has only one row of "arms" (Hopkins, 1970) Electron micrographs of 9 + 1 axonemes indicated that the central tubule having *two* rows of arms was the one which remained after detergent treatment.

When preparations of intact axonemes were placed in 0 15%–0.20% Sarkosyl, both of the central tubules were removed in most axonemes and the preparations contained primarily outer doublet tubules.

(2) OUTER DOUBLET TUBULES: It was not possible to remove both of the central tubules from a preparation of 9 + 2 or 9 + 1 axonemes without also removing some of the outer portion of the B

tubule from many of the outer doublets Therefore, to obtain a preparation of outer doublets *with little or no B tubule breakdown,* the flagella of the paralyzed mutant pf-18 were used These 9 + 0 flagella lack the two central tubules and instead contain unstructured material in the center of the axoneme (Fig 13 a) The flagella of the mutant cells were isolated by the same procedure described for isolating flagella from wild-type cells When the membranes were removed from pf-18 flagella isolated by the STEEP + calcium method (see Sec II A), the matrix and unstructured material in the center of the axoneme were solubilized and a preparation of nearly-intact outer doublet microtubules was obtained (Fig 13) These preparations were used in many of the following studies on the selective solubilization of the protofilaments of the outer doublet microtubules

When preparations of intact outer doublet tubules obtained from pf-18 flagella were treated with 0.15–0 20% Sarkosyl, the first part of the doublet to be solubilized was the outer portion of the B tubule (Fig 14). This was the same portion of the B tubule which sometimes passed into solu-
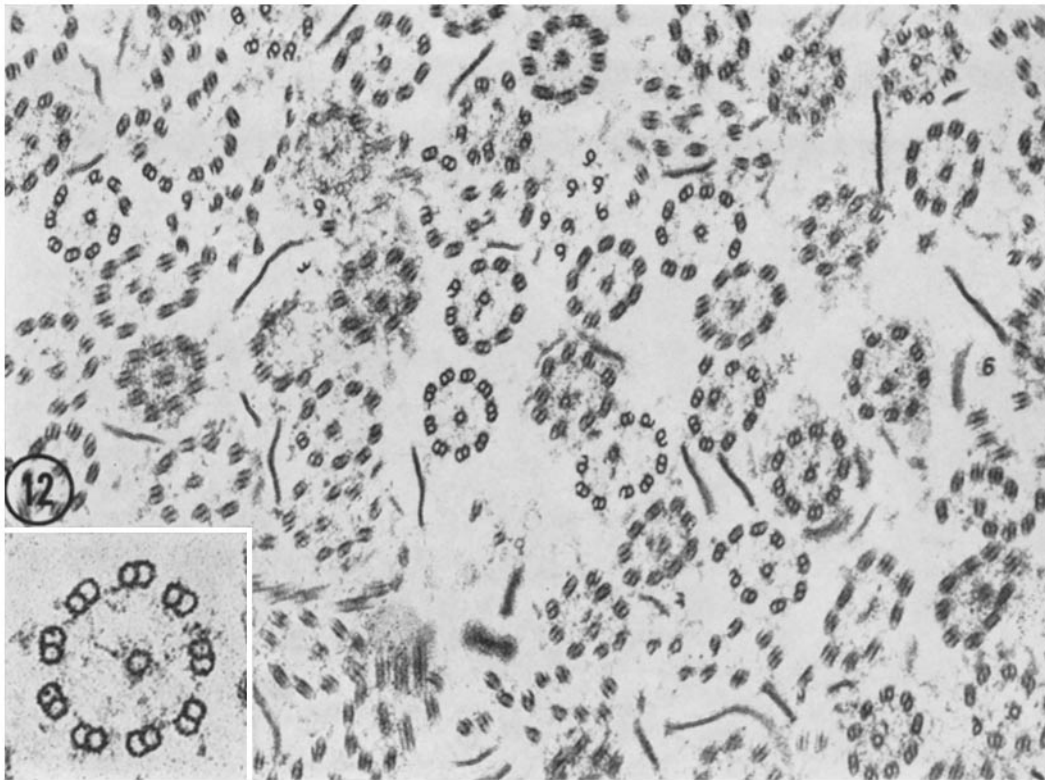
FIGURE 12 Isolated axonemes treated with 0.1% Sarkosyl. In most axonemes one of the two central tubules has been removed. In some of the outer doublets the outer portion of the B tubule has also been solubilized. × 53,000. *Inset:* × 114,000.

tion during membrane removal and/or solubilization of the two central tubules of wild-type (21-gr) flagella by dialysis or Sarkosyl treatment (see Sec. II).

When intact outer doublet tubules were treated with 0 2–0 4% Sarkosyl, almost all of the B tubule was removed, leaving preparations which contained primarily A tubules (Fig 15).

Treatment of outer doublet microtubules with 0 5–0 6% Sarkosyl selectively removed the B tubule and the outer portion of the A tubule. When examined by negative-staining procedures *the protofilaments of each partially solubilized A tubule were observed to be separating along their lengths into one group of three protofilaments and several groups of two protofilaments* (Fig. 16). Individual groups of two protofilaments ("double" protofilaments) appeared to be splitting away from the group of three protofilaments, but they were rarely observed completely separated from the group of three This ob-

servation suggested that the double protofilaments were rapidly solubilized after they became detached from the group of three protofilaments.

In contrast, the unique group of three protofilaments was relatively resistant to solubilization by Sarkosyl [5] When the outer doublets were treated with 0.7% Sarkosyl, all the double protofilaments were solubilized, while the three contiguous protofilaments persisted, even after treatment for 16 hr (Fig 17). *Such preparations consisted entirely of groups of three protofilaments, there were no groups of two or four*

Analysis of the progressive solubilization of outer doublet tubules by both thin-sectioning and negative-staining procedures indicated that these three protofilaments were the ones which formed the wall or "partition" between the lumens of the A and B tubules of the outer doublets. Fig. 18 shows

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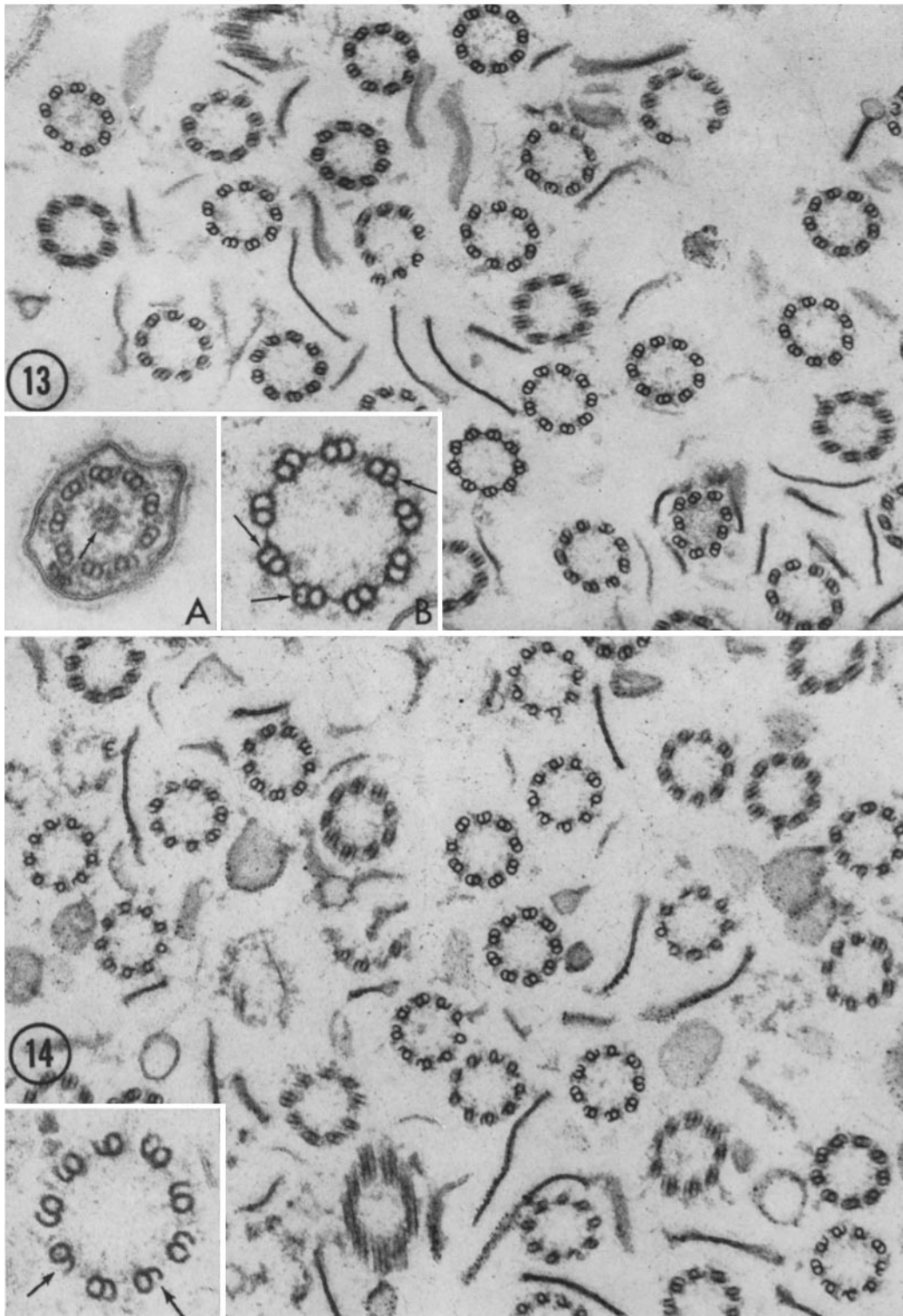[5] We thank Dr. L. Rebhun for initially bringing this group of insoluble protofilaments to our attention.

FIGURE 13   9 + 0 axonemes isolated from flagella of the paralyzed mutant pf-18 by the STEEP minus calcium rinse procedure. × 53,000. *Insets: A, In situ* whole flagellum of mutant pf-18. The two central tubules are replaced by an apparently structureless core (arrow). × 84,000. *B*, Isolated 9 + 0 axonemes also have the peripheral links between the outer doublets and the beaklike projections which extend from the wall of the B tubule into the lumen of that tubule (arrows). × 128,000.

FIGURE 14   Isolated 9 + 0 axonemes treated with 0.15% Sarkosyl. In most axonemes the outer portions of the B tubules have been solubilized (arrows, *inset*) while the A tubules remain intact. × 54,000. *Inset:* × 116,000.
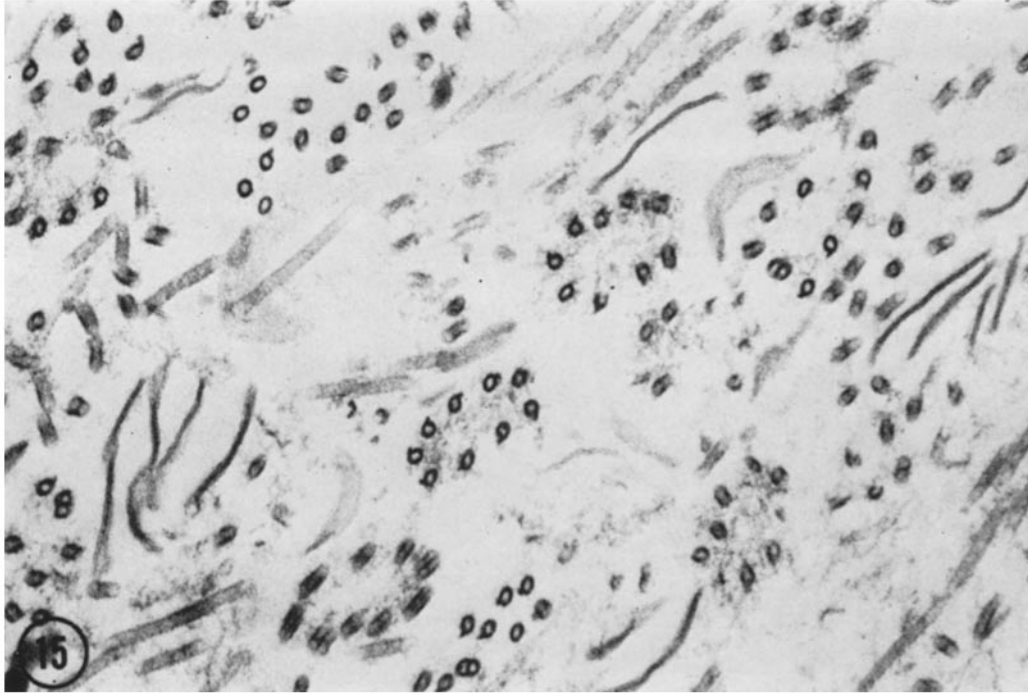
FIGURE 15   A tubules obtained from isolated 9 + 0 axonemes by treatment with 0.3% Sarkosyl. In most outer doublets the B tubule has been almost completely solubilized, while the A tubule still remains relatively intact. × 74,000.

several outer doublets which have been partially solubilized at one end The partitions between the lumens of the A and B tubules are visible as electron translucent stripes (bars, Fig 18) running longitudinally down the centers of the outer doublets. Each of these stripes is bordered by two darker stripes which are the stain-filled lumens of the A and B tubules. At the ends of the outer doublets all of the protofilaments of the A and B tubules have been solubilized with the exception of the protofilaments comprising the partition (arrows, Fig 18) Some of these partitions can be observed to contain three protofilaments These observations, in addition to others made on thin-sectioned material, establish the identity of the Sarkosyl-insoluble group of three protofilaments with the partition between the lumens of the A and B tubules of the outer doublets [6]

Although the selective solubilization of the

[6] These observations support the model preferred by Ringo (1967 b, Fig. 2 h) for subunit arrangment in the outer doublets of *Chlamydomonas* flagella In this model, three protofilaments form the partition between the lumens of the A and B tubules.

axonemal microtubules described above was carried out by treating *intact* 9 + 2 or 9 + 0 axonemes with Sarkosyl, a preparation of axonemes could be treated *successively* with increasing concentrations of the detergent to obtain, for example, A tubules, from which a preparation of the three partition protofilaments could then be obtained However, successive solubilizations carried out on the same preparation were sometimes difficult to control and the preparations were less uniform Therefore, for most studies, it was more practical to treat the intact 9 + 2 or 9 + 0 axonemes with the concentration of detergent known to give the required degree of solubilization

(C) FRACTIONATION OF AXONEMES BY HEAT TREATMENT

Stephens (1970 a) has described methods which utilize brief heat treatments at temperatures of about 40°C for the selective solubilization of outer doublet microtubules of sea urchin sperm flagella Similar procedures could also be used to selectively solubilize some of the microtubules of *Chlamydo-*
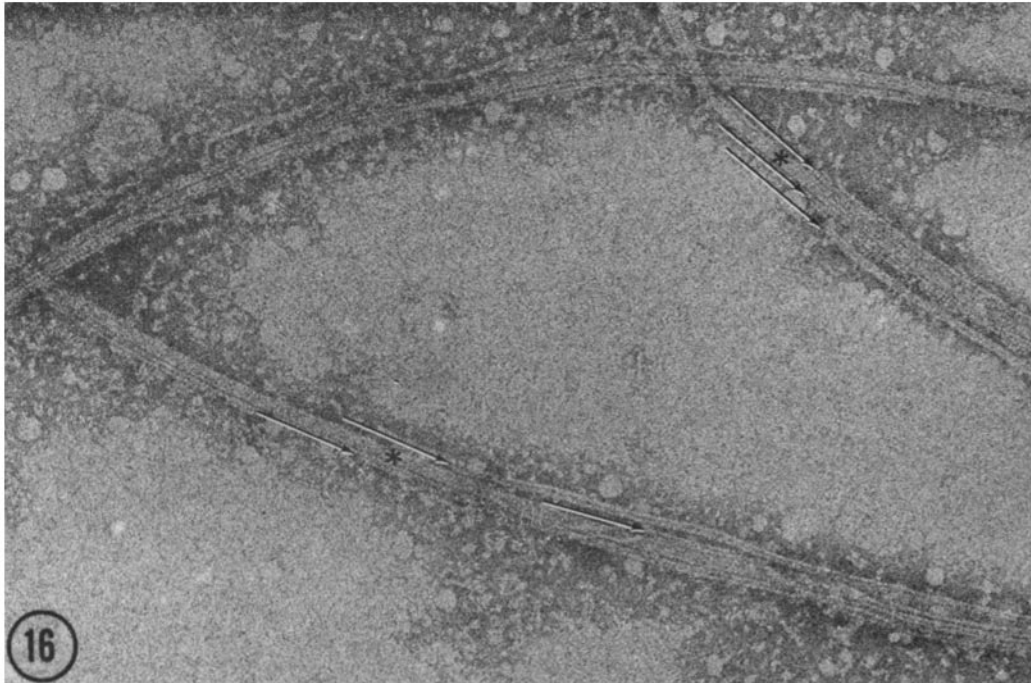
FIGURE 16 Electron micrograph of A tubules partially solubilized with 0.6% Sarkosyl and negatively stained with PTA. The individual protofilaments of the microtubules are readily apparent; each microtubule contains one group of three protofilaments (asterisks) and several groups of two protofilaments (arrows). The groups of protofilaments have separated along their lengths. (The groupings become more apparent when the micrograph is tilted so that the viewer sights along the length of the tubule.) × 105,000.

monas axonemes. For example, if intact 9 + 2 axonemes isolated by the STEEP minus calcium rinse procedure were resuspended in 3.0 mM MgSO₄, 10 mM Tris (pH 7 1 at 46°C), and agitated for 5–6 min at 46°C, one of the two central tubules was preferentially removed from 50–75% of the axonemes When outer doublet tubules isolated from sucrose-pH flagella by the TEM procedure were suspended in 10 mM Tris (pH 7 2 at 48°C) and agitated for 6 min at 48°C, most of the B tubules were solubilized, leaving preparations containing A tubules

The complete sequence of solubilization of the microtubules of 9 + 2 and 9 + 0 axonemes by Sarkosyl and heat treatments is summarized diagrammatically in Fig 19

(VII) *Ultrastructural Observations*

(A) INTRATUBULAR STRUCTURES

A beaklike projection extending from the wall of the B tubule into the lumen of that tubule was

observed in three of the nine outer doublets in many cross-sections of whole flagella (Fig 3, inset) and of isolated 9 + 2 (Fig. 10 b) and 9 + 0 (Fig 13 b) axonemes of wild-type and mutant cells. Analysis of many hundreds of cross-sections of isolated 9 + 2 axonemes indicated that (a) these structures almost always occurred in two adjacent B tubules on one side of the axoneme and in one B tubule on the opposite side of the axoneme, and (b) this constant 2:1 positioning was variable with respect to the plane of the two central tubules (see Discussion)

(B) PERIPHERAL LINKS

Peripheral links between adjacent outer doublets were observed in cross-sections of isolated 9 + 2 (Fig 10 a) and 9 + 0 (Fig 13 b) axonemes of wild-type and mutant *Chlamydomonas*. Each link appeared as a straight, rodlike structure extending from the wall of an A tubule to the wall of the adjacent B tubule, where the link terminated in a
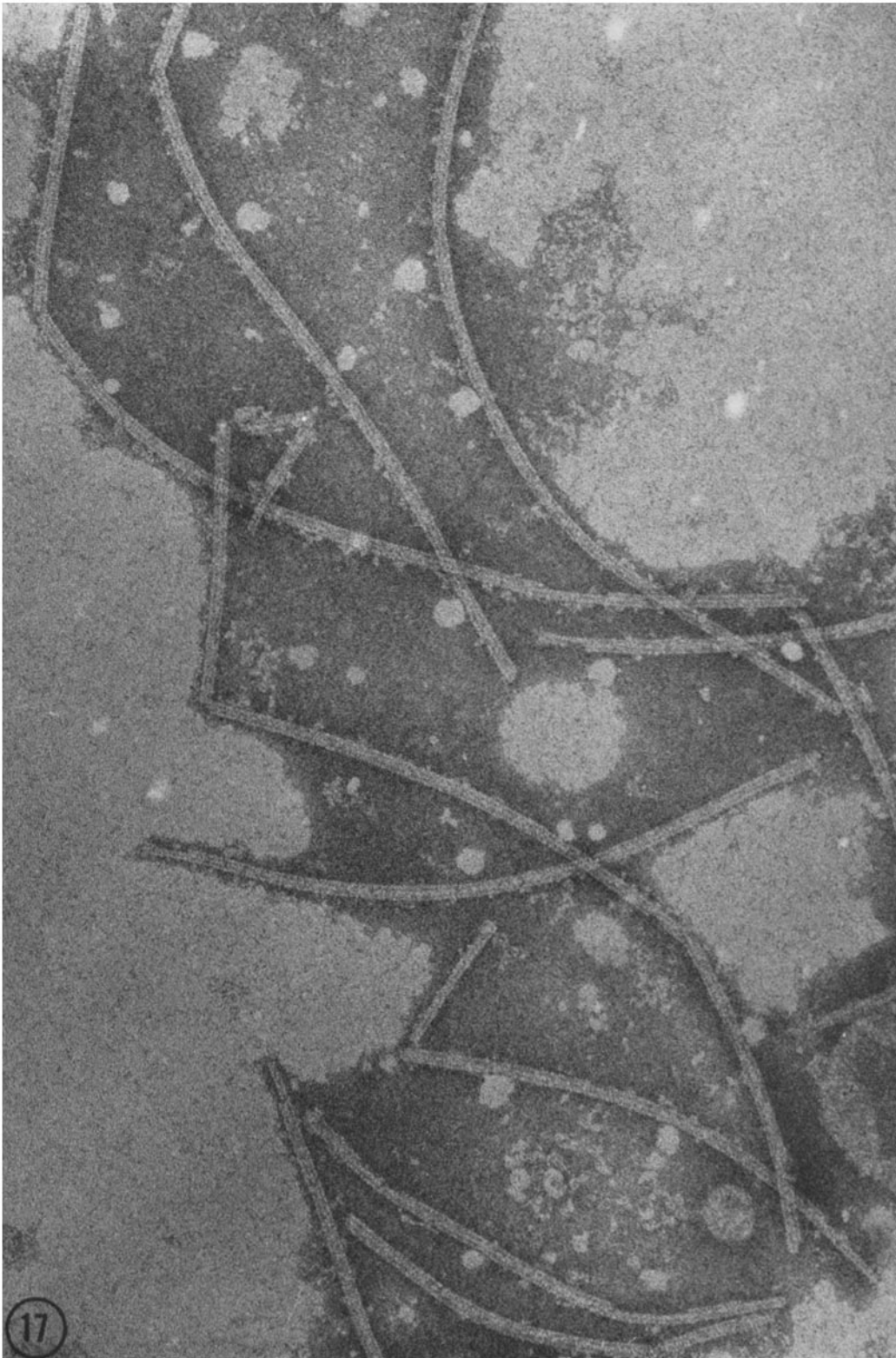
FIGURE 17   Electron micrograph of negatively stained partition protofilaments remaining after treating isolated outer doublets with 0.7% Sarkosyl for 16 hr. Each partition contains three protofilaments. × 116,000.

FIGURE 18 Electron micrograph of partially solubilized outer doublet microtubules. In each outer doublet the partition between the dark, stain-filled lumens of the A and B tubules appears as a white stripe (bars) In the lower part of the micrograph, most portions of the outer doublets have been solubilized with the exception of the partitions (arrows), some of which appear to contain three protofilaments. (These features are more apparent when the micrograph is tilted so that the viewer sights along the arrows.) × 135,000.

slight thickening of the B tubule wall These links are 180–190 A long

(VIII) Electrophoretic Analysis of the Flagellar Components

(A) UREA ACRYLAMIDE GELS

The protein composition and purity of the various flagellar fractions were determined by quantitative urea acrylamide gel electrophoresis (see Materials and Methods) By determining which peaks on densitometric scans of the acrylamide gels were enriched as a certain component was purified (as determined by electron microscope observations), specific proteins could be assigned to certain flagellar structures. In addition, by comparing the areas of the appropriate regions of the densitometric scans of the gels, the relative purity of the various fractions could be determined

(1) WHOLE FLAGELLA: When the proteins of unfractionated 9 + 2 flagella were separated on urea acrylamide gels, the pattern shown in the gel (fast green stain) and corresponding densitometric scan in Fig. 20 was obtained. The two prominent peaks represented the two microtubule proteins (the evidence for the microtubule nature of these proteins is presented below) The microtubule protein in gels such as these represented ca 40% of the total protein in the gel In addition, a number of minor protein bands were observed in these gels, these were particularly apparent when the gels were stained with the more sensitive Coomassie blue rather than with fast green (Fig 22)

(2) MATRIX· Matrix fractions obtained by dialyzing sucrose-pH flagella against TEM were greatly enriched for some of the minor proteins Fig. 21 shows a fast green-stained gel of a typical matrix fraction, representing ca 35–40% of the total flagellar protein Gels of this fraction contained many prominent bands which corresponded to relatively minor bands in fast green-stained gels of whole flagella Some of these bands probably represented the proteins of such extramicrotubular structures of the axonemes as the radial and peripheral links, since these structures were preferentially solubilized by dialysis of sucrose-pH flagella against TEM, other bands were probably from the proteins constituting the amorphous flagellar matrix, which was also solubilized by the TEM fractionation procedure Together, these "minor" proteins comprised more than 80% of the total

protein in a gel of the matrix fraction. The micro-
tubular proteins comprised only about 15–18% of
the protein, their presence was probably due to the
solubilization of most of the central tubules and a

small amount of the B tubule during the TEM
dialysis procedure.

Comparison of gels of the axoneme and matrix
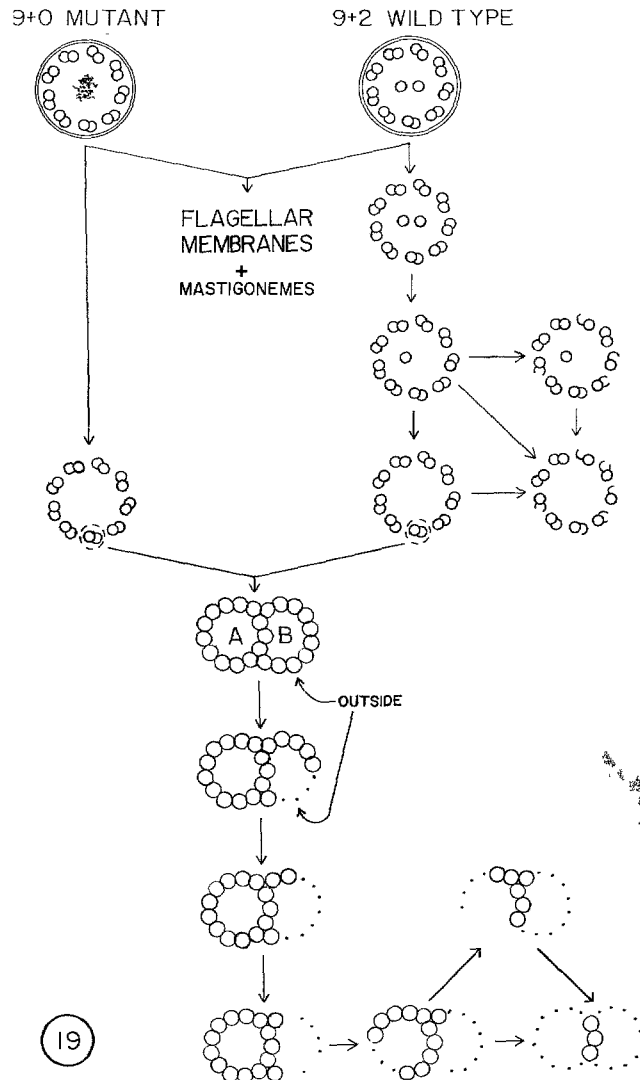fractions obtained by rinsing flagella in STEEP

Figure 19   Illustration of the sequence of solubilization of microtubules from 9 + 0 flagella of mutant
cells and from 9 + 2 flagella of wild-type cells. Removal of membranes from 9 + 0 flagella resulted in
preparations of intact outer doublets; removal of membranes from 9 + 2 flagella left 9 + 2 axonemes
containing nine outer doublets and two central tubules. When 9 + 2 axonemes were treated with in-
creasing concentrations of Sarkosyl, one of the central tubules was removed; then the remaining central
tubule was solubilized, often along with the outer portion of some of the B tubules. Intact outer doublets
from either 9 + 0 or 9 + 2 axonemes were then solubilized in the following sequence by treatment with
increasing concentrations of Sarkosyl: the outer portion of the B tubule; the remaining portions of the B
tubule; the outer portion of the A tubule; the remainder of the A tubule with the exception of the three
partition protofilaments.
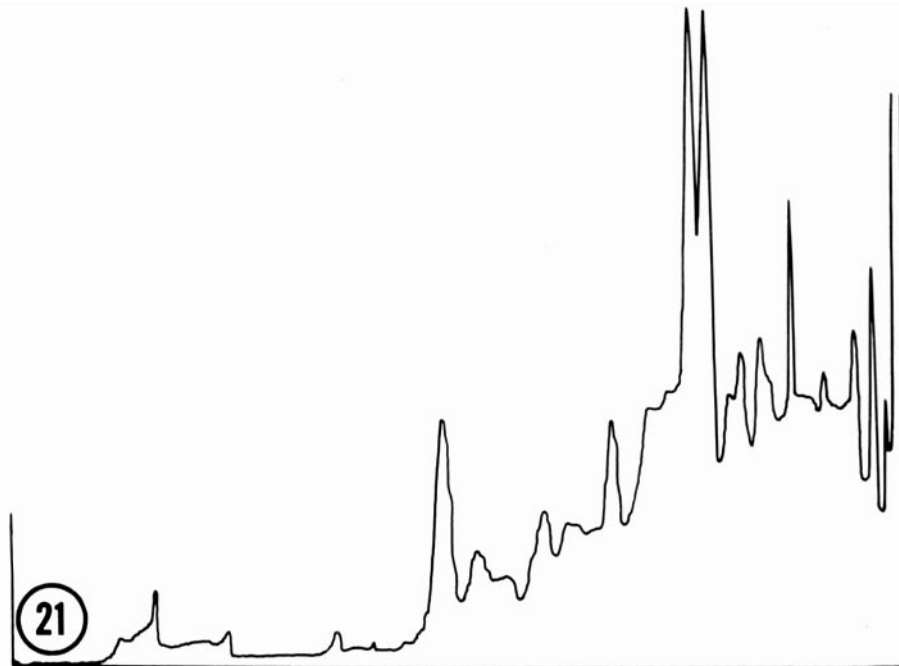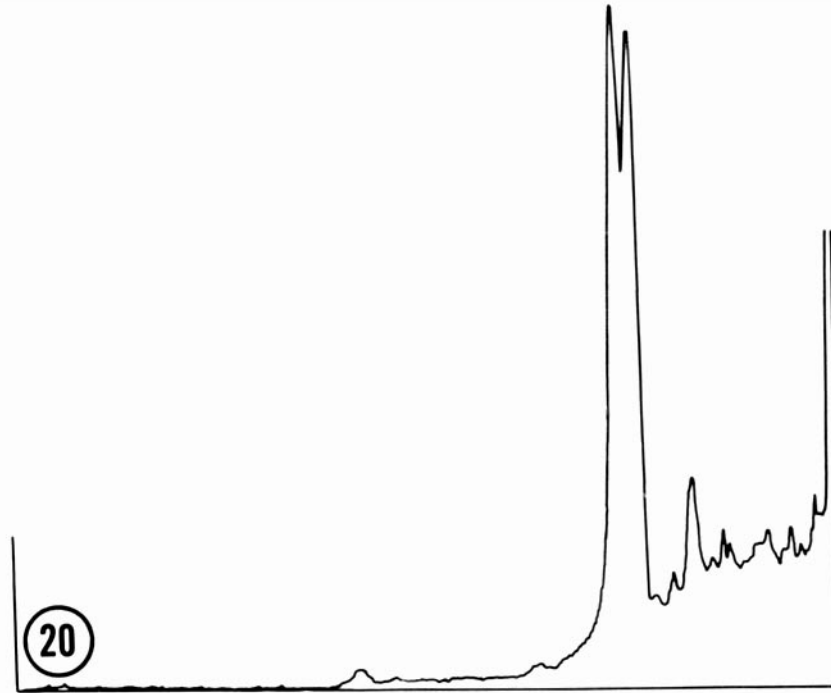
FIGURES 20 and 21   Fast green-stained urea acrylamide gels with their corresponding densitometric tracings. Fig. 20, Whole flagella. Fig. 21, Matrix from TEM dialysis of sucrose-pH flagella.
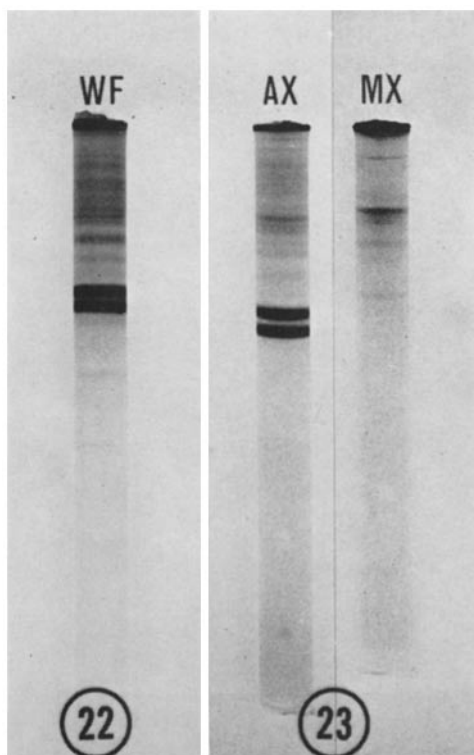
529

Figure 22 Whole flagellar (*WF*) proteins separated on urea acrylamide gels and stained with Coomassie blue.

Figure 23 Fast green-stained urea acrylamide gels of intact 9 + 2 axonemes (*AX*) and matrix (*MX*) obtained by the STEEP minus calcium rinse procedure.

the electrophoretic patterns of whole flagella (Fig 20). Since 80–90% of the total protein in gels of purified outer doublets was contained in these bands, the outer doublets must be composed primarily of these two proteins. Fast green staining showed that the two microtubule proteins were present in approximately equal quantities in the outer doublets.

As shown in Fig 25, these two microtubule proteins could be separated even further by increasing the time of electrophoresis This separation facilitated the removal of each microtubule protein from the gels for use in biochemical analyses described elsewhere (Olmsted et al, 1971, Carlson, Witman, and Rosenbaum, in preparation)

(4) CENTRAL MICROTUBULES. As noted above, the two microtubule proteins constituted ca 15–18% of the total protein in gels of the matrix fraction obtained by dialysis of 9 + 2 flagella against TEM. Since this fractionation procedure solubilized the central tubules while leaving the outer doublets relatively intact (see Sec VII A), the presence of the two microtubule proteins in the matrix fraction suggested that the central microtubules were composed of the same two proteins as the outer doublet microtubules To substantiate this, the protein composition of intact 9 + 2 axonemes (Fig 10) obtained from flagella of wild-type cells by the STEEP minus calcium rinse procedure was compared to that of 9 + 0 axonemes (Fig. 13) obtained from flagella of mutant cells by the same procedure The 9 + 2 and 9 + 0 axonemes were found to contain the two microtubule proteins *in the same ratios* when analyzed by quantitative urea acrylamide gel electrophoresis (Fig 26) This result verified that *both* of the microtubule proteins were contained in the two central tubules If the two central tubules had been made up of only *one* of the proteins, the ratio of the proteins in 9 + 2 axonemes having the two central tubules would have differed by approximately 20% from the ratio in 9 + 0 axonemes lacking the two central tubules.

The only difference consistently observed between the preparations was one minor band present in gels of 9 + 2 axonemes but not in gels of 9 + 0 axonemes. This band, which contained about 8–10% as much protein as the combined microtubule protein bands, probably represented the proteins of an extramicrotubular structure present only in the 9 + 2 axonemes (see Discussion).

minus calcium indicated that relatively few of the matrix proteins were solubilized when the flagellar membranes were removed by this procedure Those that were removed (Fig 23, *MX*) probably represented proteins of the amorphous, densely-staining matrix since this appeared to be the only material solubilized by this rinse procedure, those proteins that remained with the axonemal fraction (Fig. 23, *AX*) probably represented the proteins of the links, arms, and other extramicrotubular axonemal structures since these parts could still be observed in the axonemes prepared by this rinse procedure (See Fig. 10)

(3) OUTER DOUBLET MICROTUBULES: The proteins of outer doublet microtubules (Figs 11 and 13), obtained from either 9 + 2 or 9 + 0 flagella, separated into two major bands when analyzed by urea acrylamide gel electrophoresis (Fig. 24) These proteins had the same relative mobilities as the two major proteins observed in
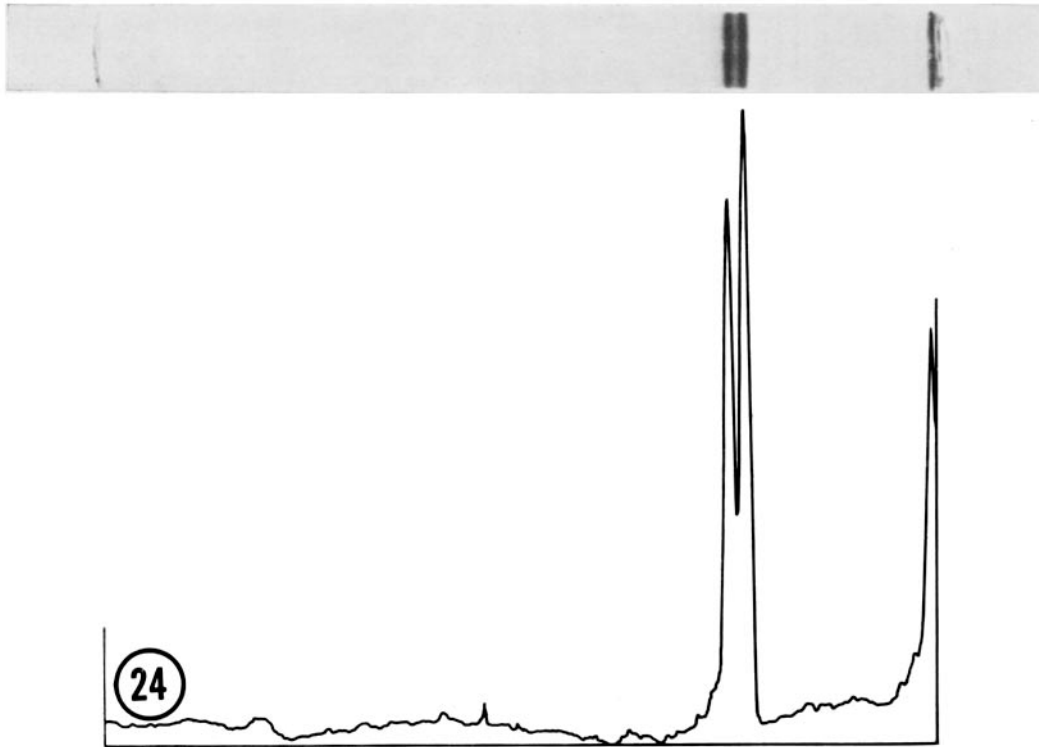
FIGURE 24 Fast green-stained urea acrylamide gel, and corresponding densitometric tracing, of outer doublets obtained by the TEM dialysis procedure.

(5) MASTIGONEMES. When purified mastigonemes were analyzed by electrophoresis on urea acrylamide gels (Fig 27, MG), only one band was observed, indicating that the mastigonemes contained a single polypeptide This band, which corresponded to one of the minor bands observed in gels of whole flagella (Fig 27, WF), was also revealed by the periodic acid-Schiff (PAS) staining procedure, which is specific for carbohydrates The protein may therefore contain covalently-bound carbohydrate. The mastigoneme fraction was free of matrix and axoneme proteins

(6) MEMBRANES· When purified flagellar membranes (prepared for electrophoresis by the same procedures used for the other flagellar fractions) were analyzed on 7.5% urea acrylamide gels, no protein bands were observed in the gels Since microtubule, matrix, and mastigoneme proteins would have entered the gels under these conditions, the membrane fraction (Fig 5) was apparently free of these nonmembrane contaminants

(B) SDS-UREA ACRYLAMIDE GELS

Whole flagella, axonemes, membranes, and mastigonemes were also analyzed on SDS-urea

acrylamide gels (Laemmli, 1970) The hot SDS treatment used to prepare proteins for this procedure has been shown to solubilize membrane (Lenard, 1970) and other structural proteins (Maizel, 1969) which resist dissociation by many other procedures (including 8 M urea treatment. cf Dickson et al, 1970). This treatment therefore insured that most, if not all, of the flagellar proteins were solubilized. In addition, the high resolution, discontinuous gel system used here has been shown to separate proteins on the basis of size, thus permitting estimations of the molecular weights of the various proteins (Olmsted et al., 1971)

(1) WHOLE FLAGELLA: When analyzed by electrophoresis on SDS-urea gels, the proteins of whole flagella separated into three major bands and a large number of minor bands (Fig 28, WF) The three major bands and certain of the minor bands were identified with particular flagellar structures by comparing the gels of whole flagella with those of the various flagellar components. As with the urea gels, such comparisons also provided an indication of the degree of purity which had been achieved in each fraction

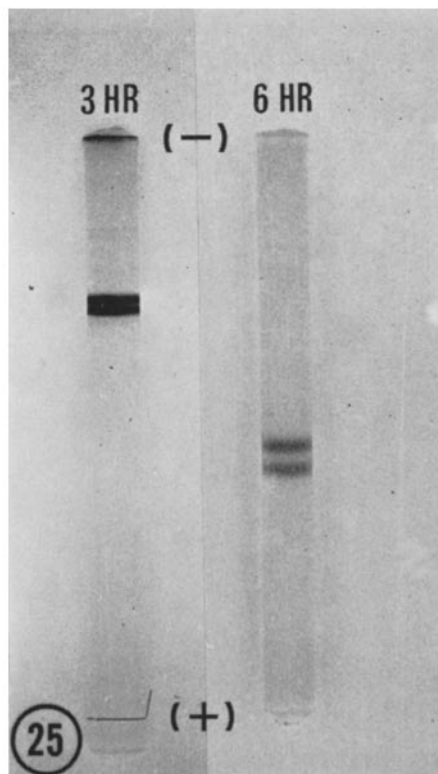(2) AXONEMES: When isolated 9 + 2 axo-

FIGURE 25 Proteins of outer doublet microtubules separated on urea acrylamide gels by electrophoresis at 3 ma/gel for 3 hr and 6 hr. Fast green stain.

nemes (Fig. 10) were analyzed by electrophoresis on SDS-urea gels (Fig. 28, *AX*), most of the protein in the gel migrated as two closely moving bands having the same relative mobilities as two of the three major bands observed in gels of whole flagella These two bands represented the two proteins of the flagellar microtubules, since no other structures were present in sufficient quantity in the axoneme fraction to account for them. Comparison of the relative mobilities of these proteins with those of standards of known molecular weights indicated that the microtubule proteins had molecular weights of 53,000 and 56,000 daltons (Olmsted et al., 1971) SDS-urea gels of 9 + 2 axonemes also contained a large number of minor bands, which probably represented the proteins of extramicrotubular structures (see Sec VIII A 2).

(3) MEMBRANES: Isolated membranes dissociated with hot SDS contained one major protein when analyzed by electrophoresis in SDS-urea gels (Fig 28, *MB*). This protein migrated with the

same relative mobility as the slowest moving of the three major bands observed in gels of whole flagellar proteins (Fig. 28, *WF*). A very small amount of the membrane protein routinely co-migrated with the tracking dye The almost complete absence of microtubule and mastigoneme protein bands indicated that the membrane fraction was relatively free of these flagellar structures

(4) MASTIGONEMES: A single band was observed when isolated mastigonemes were analyzed on SDS-urea gels (Fig. 28, *MG*), confirming that mastigonemes contained only one protein, and that the preparations of purified mastigonemes were free of membrane, axoneme, and matrix proteins. Comparisons of the relative mobility of this protein (which is probably complexed with carbohydrate, see above Sec. VIII A 5) with those of standards of known molecular weights indicated that the mastigoneme subunit had a molecular weight of approximately 170,000 daltons.

(C) SUMMARY OF ELECTROPHORETIC RESULTS

The flagella were found to contain two microtubule proteins, which composed both the outer doublet and central microtubules, and a large number of minor proteins, many of which probably made up extramicrotubular structures such as the peripheral and radial links of the axoneme. Most of these minor proteins were selectively obtained in the matrix fraction The membrane fraction contained one major protein band in SDS-urea gels, and was relatively free of microtubule, matrix, and mastigoneme components. The mastigoneme fraction was found to contain only a single glycoprotein

DISCUSSION

In this report, methods have been described for the isolation of *Chlamydomonas* flagella, for their fractionation into membrane, mastigoneme, matrix, and microtubule components, and for the subfractionation of the outer doublet microtubules Each fraction has been studied by electron microscopy and by electrophoresis on two different acrylamide gel systems.

Fractionation of Flagellar Axonemes

When the axonemes were treated with increasing concentrations of the detergent Sarkosyl, the
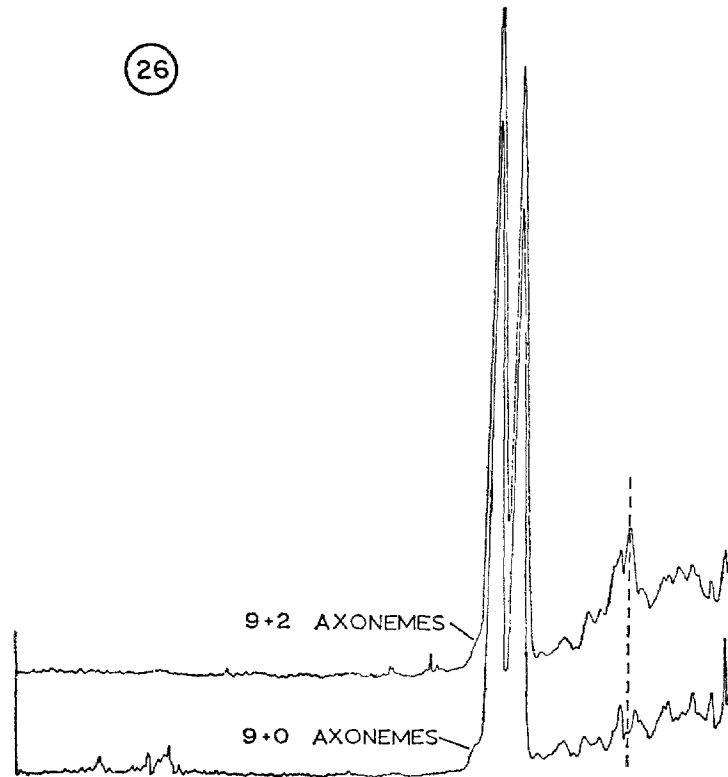
Figure 26 Densitometric tracings of fast green-strained urea acrylamide gels of 9 + 2 axonemes from flagella of wild-type cells and 9 + 0 axonemes from flagella of mutant cells The two microtubule proteins (large peaks) are present in the same relative amounts in 9 + 2 axonemes as in 9 + 0 axonemes. One minor peak (dashed line) is present in 9 + 2 axonemes but not in 9 + 0 axonemes.

microtubules were solubilized in the following order: one of the two central tubules, the remaining central tubule and the outer parts of the B tubules, most of the remaining portions of the B tubules, the outer portions of the A tubules, the remaining (inner) portions of the A tubules with the exception of the three protofilaments which form the partition between the A and B tubules This sequence of solubilization could be defined because one could distinguish the A tubule from the B tubule as well as the inner and outer portions of the tubules This was possible because the outer doublets tended to remain in their normal circular association during the solubilization procedures In STEEP + calcium-isolated axonemes, for example, almost all of the B tubules could be removed while the A tubules still remained in a circle (Fig. 15) This permitted one to observe the sequence of solubilization of the A tubule starting from the outer portions, until only the three par-

tition protofilaments remained. In many cases, at stages before obtaining the three remaining protofilaments, the partitions could be observed to be attached to one or two of the innermost protofilaments of both the A and B tubules (Fig 19) These observations, along with those on negatively-stained preparations (Fig 18) therefore enabled the unequivocal localization of the three remaining protofilaments as those forming the partition between the A and B tubules.

The differential solubilities of the axonemal microtubules might have been due to (a) the different positions of the microtubules in the axoneme, (b) the presence of various extramicrotubular structures attached to the different tubules, or (c) biochemical differences between the subunit proteins of the tubules

The relative locations of the various microtubules within the axoneme could account for a few of the observed differences in solubility For
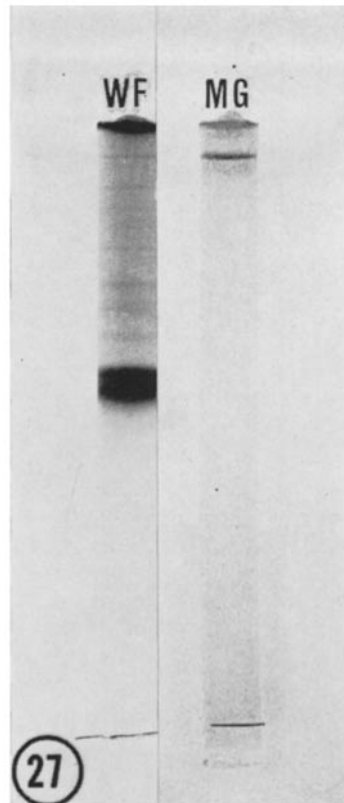
FIGURE 27 Whole flagella (WF) and isolated mastigoneme (MG) proteins stained with fast green after electrophoresis in urea acrylamide gels. (Both gels contained 5% acrylamide; the two microtubule protein bands do not separate as well in these 5% gels as in 7.5% acrylamide gels.)

FIGURE 28 Whole flagella (WF), axoneme (AX), mastigoneme (MG), and membrane (MB) proteins after electrophoresis on SDS-urea acrylamide gels. The membrane (mb), mastigoneme (mg), and microtubule (mt1 and mt2) protein bands in the whole flagella gel are marked. (MG is a 5% acrylamide gel; the others are 7.5% acrylamide gels.)

example, the high stability of the inner wall relative to the outer wall of either the A or B tubule might have been due to the relative positions of these walls within the axoneme, if portions of microtubules nearer to the periphery of the axoneme were more susceptible to detergent treatment than those portions closer to the center of the axoneme However, such positional effects could not explain why one of the two central tubules was solubilized almost simultaneously with the outer wall of the B tubule, or why one of the central tubules was solubilized before the other, even though the two tubules occupied the same relative positions in the axoneme

Roth et al , (1970) have hypothesized that the binding of intermicrotubular structural elements to microtubule subunits might stabilize that por-
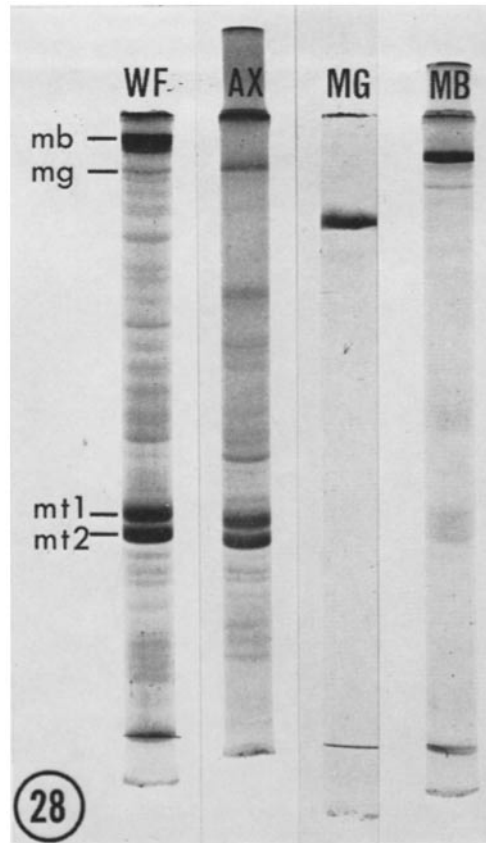
tion and neighboring parts of the microtubule. Some of the differences observed in microtubule solubility might be attributed to such effects For example, Hopkins (1970) has shown that in Chlamydomonas flagella one of the two central tubules appears to have two rows of arms while the other has only one. It was observed in the present study that the central tubule having two arms was the more stable of the two central tubules Also, the A tubule of the outer doublet has radial and peripheral links and two rows of arms (dynein) attached to it, the attachment of these elements might account for the observed stability of the A tubule relative to the B tubule, to which are at-

tached only peripheral links Furthermore, in both the A and B tubules, most of the secondary structures are attached to that half of the tubule nearest the central tubules, this portion of both the A and B tubules was more stable than the outer half of the tubule

Most, if not all, of the observed differences in microtubule solubility might therefore be accounted for by either differences in the relative positions of the microtubules within the axoneme or differences in their associations with various extramicrotubular structures However, the possibility still remains that some of the solubility differences might have been due to biochemical differences between the microtubules.

Support for the hypothesis that different microtubules and portions of the same microtubule may differ chemically has come from the studies of Behnke and Forer (1967) on the effects of temperature and pepsin on the solubilization and digestion of the microtubules of crane-fly spermatids and rat sperm tails They found that the cytoplasmic microtubules, central microtubules, B tubules, and A tubules were affected differently by these treatments, and that some parts of the B tubule were more stable than other parts of that tubule The sequence of solubilization of the microtubular axoneme which they reconstructed from their studies (their Fig 1) has several similarities to the sequence of solubilization of isolated flagellar microtubules described in this report (cf Fig 19) They concluded that the different types of microtubules had "intrinsic physical and/or chemical differences" and that portions of the B tubule wall adjacent to the A tubule were compositionally different from the remaining portions

Similarly, Kiefer (1970), studying the in vivo degeneration of sperm in a sterile mutant of Drosophila, found that the A and B tubules differed from each other and from the central tubules in their patterns of breakdown, and that portions of the walls of the A and B tubules were lost in a definite sequence Kiefer hypothesized that the central tubules and the inner and outer walls of both the A and B tubules may be chemically different.

These studies suggest that there may indeed be biochemical differences between different types of microtubules and also between different regions of a single microtubule This possibility is considered in the following report, in which microtubule protein from isolated outer doublets, A

tubules, solubilized B tubules, and partition protofilaments is analyzed by electrophoresis.

Electrophoretic Analysis of Axoneme Components

OUTER DOUBLETS· Renaud et al, (1968) determined by acrylamide gel electrophoresis that the outer doublet microtubules of *Tetrahymena* cilia were composed of two major proteins The results reported here showed that the isolated outer doublet microtubules of *Chlamydomonas* flagella also separated into two major proteins when analyzed by urea and SDS-urea acrylamide gel electrophoresis, and that the proteins were present in approximately equal amounts in intact outer doublets. The two microtubule proteins have been separated, purified, and shown to have different molecular weights and amino acid contents (Olmsted et al, 1971, Carlson, Witman, and Rosenbaum, in preparation)

MATRIX When proteins of whole flagella or intact axonemes were analyzed by urea or SDS-urea acrylamide gel electrophoresis, 15–20 minor proteins were observed. Since the flagella or axonemes were treated with 8 M urea and then reduced and alkylated, most of these minor bands probably represented individual polypeptides rather than dimers or higher polymers of a few proteins Comparison of the electrophoretic patterns of whole flagella with the patterns of intact 9 + 2 axonemes, isolated outer doublets, and matrix fractions indicated that many of these minor bands represented structures such as the arms (dynein) of the outer doublets, the arms of the central tubules, the peripheral and radial links, and other nonmicrotubular elements of the flagellum (see Allen, 1968, Warner, 1970, Hopkins, 1970 as well as Figs 10 a and 13 b in this report) Further correlation of *specific* protein bands with *specific* structures should be possible by comparing the electrophoretic patterns of flagella of wild-type cells with those of mutants lacking various structural components, or by isolating the different structures and analyzing each by electrophoresis, as was done in this study with the flagellar microtubules, membranes, and mastigonemes

CENTRAL TUBULES: That the central microtubules contained the same two proteins as the outer doublets was suggested by the findings that (a) two proteins having the same relative mobilities as the proteins of the outer doublets were

present in matrix fractions containing the solubilized proteins of the central tubules, and (b) these two proteins were present in the same ratios in 9 + 2 axonemes containing the two central tubules as in 9 + 0 axonemes lacking the central pair Although one minor band was present in gels of 9 + 2 axonemes and not in gels of 9 + 0 axonemes, it is very unlikely that this band could have represented the proteins of the central tubules, since (a) it contained less than 10% of the protein of the combined microtubule protein bands, while the central pair should contain nearly 13% as much protein as the outer doublets, and (b) microtubule proteins from a number of different sources have all been shown to have similar electrophoretic mobilities (Olmsted et al , 1971) This band probably represented the protein of the arms or sheath of the two central tubules, since these structures were present in 9 + 2 axonemes but not in 9 + 0 axonemes (cf Figs. 10 and 13).

Membranes

Preparations of flagellar membranes were nearly free of other flagellar components as judged by electron microscopy and acrylamide gel electrophoresis They were also free of other types of cell membranes, since the flagella were isolated without rupturing the cell bodies These flagellar membrane fractions therefore provide excellent material for biochemical studies of a specific membrane type, for the investigation of cell membrane morphogenesis in conjunction with systems of regenerating flagella (Rosenbaum et al , 1969), and for the study of the possible role of flagellar membranes in gamete recognition and agglutination (see Wiese, 1970 for review).

When isolated membranes were analyzed by electrophoresis on SDS-urea acrylamide gels, one major protein band was observed This protein migrated well behind the 170,000 dalton mastigoneme protein, indicating that it had a molecular weight considerably greater than 170,000 daltons (see following section). This flagellar membrane protein may be homologous to the protein of ca. 200,000 daltons reported by Gwynne and Tanford (1970) to comprise a major portion of human erythrocyte membranes, or to those proteins of ca. 240,000 and 255,000 daltons shown by Lenard (1970) to comprise about one third of the total membrane protein of various mammalian erythrocytes.

A small amount of membrane protein migrated with the tracking dye, indicating that it had a very low molecular weight. This protein may represent a proteolytic breakdown product of the major membrane protein, although proteolysis should be minimal as the cells are not ruptured during flagellar membrane isolation

Mastigonemes

The isolated mastigonemes of *Chlamydomonas* flagella were of uniform length and appeared to be composed of a single row of large subunits attached end to end The structure of these mastigonemes is therefore unique among those already described (see Bouck, 1972, Manton, 1965; and Pitelka and Schooley, 1955 for reviews) However, the mastigonemes of some other species of Chlorophyta, such as *Chlorochitridion tuberculata* (*Pedimonas tuberculata*) (Manton and Parke, 1960) and especially *Pedimonas minor* (Ettl and Manton, 1964), are very similar in size to those of *Chlamydomonas*, and may be found to have a similar ultrastructure when examined more closely

Electrophoretic analysis of isolated mastigonemes indicated that they contained a single glycoprotein having a molecular weight of ca 170,000 daltons. However, this value should be accepted only tentatively, because Segrest et al , (1971) have reported that some glycoproteins do not migrate in SDS acrylamide gels at rates proportional to their true molecular weights The only previous biochemical study of isolated mastigonemes is that of Bouck (1971), who reported that the mastigoneme protein of the Chrysophyte *Ochromonas* migrated predominately as a single band in 8 M urea gels.

The functions of the mastigonemes of *Chlamydomonas* are not known, although it has been suggested that they may be involved in motility by enlarging the effective surface area of the flagellum, thus increasing the efficiency of the flagellar stroke (Bouck, 1972, and see Pitelka, 1963) Recent findings (Witman, Bouck, and Rosenbaum, unpublished results) have indicated that the mastigonemes or mastigonemes together with the flagellar membranes are involved in the species-specific flagellar agglutination which occurs during mating of *Chlamydomonas* (see Wiese, 1970 for review). Further investigations designed to elucidate the possible role of the mastigonemes and/or the membranes in the fertilization process are in progress.

Other Inter- and Intramicrotubular Structures

INTRAMICROTUBULAR STRUCTURE: In cross-sections of outer doublet microtubules, a beaklike projection was often observed protruding from the wall of the B tubule into the lumen of that tubule. These projections almost always occurred in one outer doublet on one side of an axoneme and in the two outer doublets on the opposite side of the axoneme. Reexamination of previously published micrographs of *Chlamydomonas* flagella (Ringo, 1967 a, Fig 33, and Ringo, 1967 b, Figs 7 and 9, Jacobs and McVittie, 1970, Figs 1 and 3) showed this structure to be consistently present in the same pattern as observed in the present study, although it had not been mentioned by any of these authors. Since most of the present observations were made on isolated axonemes it was not possible to determine the longitudinal disposition of these structures in the tubule, or how the 2:1 pattern might vary in relation to the two central tubules. Such features of this new structure will have to be determined from observations of serial cross-sections of flagella *in situ*

PERIPHERAL LINKS: A straight, rodlike link connecting the A tubule of one outer doublet with the B tubule of the adjacent outer doublet has already been described in *Tetrahymena* cilia *in situ* (Allen, 1968; Williams and Luft, 1968) and in *Chlamydomonas* flagella *in situ* (Ringo, 1967 a). In the present study, similar peripheral links were observed very clearly in isolated axonemes Whether these structures were confined to any particular region of the flagellum could not be determined from observations on isolated axonemes, but the fact that they could be clearly observed in only a few cross-sections of a population of axonemes suggests that this is the case Indeed, Ringo (1967 a) observed peripheral links only in that proximal portion of the flagellum which was contained within the collar of the cell wall These peripheral links are probably not analogous to the links which are said to connect the A tubules of adjacent outer doublets in *Tetrahymena* (Gibbons, 1965) and other organisms, and which are reported to have been isolated from Echinoderm flagella (Stephens, 1970 b)

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