

Radial Spokes of *Chlamydomonas* Flagella: Polypeptide Composition and Phosphorylation of Stalk Components

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ABSTRACT Polypeptides from flagella or axonemes of *Chlamydomonas reinhardtii* were analyzed by labeling cellular proteins by prolonged growth on ^{35}S -containing media and using one- and two-dimensional electrophoretic techniques which can resolve >170 axonemal components. By this approach, a paralyzed mutant that lacks axonemal radial spokes, *pf14*, has been shown to lack 17 polypeptides in the molecular weight range of 20,000 to 124,000 and in the isoelectric point range of 4.8–7.1. Five of those polypeptides are also missing in the mutant *pf-1* which lacks only radial spokeheads. The identification of the 17 polypeptides missing in *pf-14* as components of radial spoke structures and the localization of the polypeptides lacking in *pf-1* within the spokehead, are supported by experiments of chemical dissection of wild-type axonemes. Extraction procedures that solubilize outer and inner dynein arms preserve the structure of the radial spokes along with the 17 polypeptides in question. Six radial spoke polypeptides are solubilized in conditions that cause disassembly of radial spokeheads from the stalks and those components include the five polypeptides missing in *pf-1*. No Ca^{++} - or Mg^{++} -activated ATPase activities were found to be associated with solubilized preparations of wild-type radial spokeheads. In vivo pulse ^{32}P incorporation experiments provide evidence that >80 axonemal components are labeled by ^{32}P and that five of the radial spoke stalk polypeptides are modified to different extents.

Eukaryotic cilia and flagella are complex organelles that retain their intrinsic motility when separated from cells. In most cases, their core structure, the axoneme, consists of nine doublet microtubules forming a cylinder that surrounds a pair of central microtubules. Attached to these continuous structures are appendages that occur at precise longitudinal intervals. On the A subfiber of the outer doublet are attached inner and outer dynein arms, interdoublet links, and radial spokes; the latter extend toward projections attached to the central pair (for review, see reference 1).

For several years our laboratory has been engaged in genetic, morphological, and biochemical studies of flagellar motility mutants of *Chlamydomonas reinhardtii* as a means of dissecting the complex structure and function of the flagellar axoneme (2–7). In this communication we report the analysis of the polypeptides that compose the radial spokes.

Although radial spokes in different organisms differ somewhat in ultrastructure and in longitudinal periodicity, they are

invariably periodic structures differentiated into a basal stalk segment and a distal head portion. In wild-type *Chlamydomonas* axonemes, as previously described (2, 8), the radial spokes occur in pairs with an alternate period of 29 and 62 nm. The spoke stalk measures ~32 nm in length and terminates in a bulbous enlargement, the “spokehead.” In negative stain images the heads of two spokes within a pair are sometimes seen to be connected by a thin fiber.

Independent lines of evidence indicate that the radial spokes may play an important role through interaction with the central pair microtubule complex to convert sliding of the outer doublet microtubules into bending (1, 8, 9).

For the study of the radial spoke, our approach has been to analyze flagellar and axonemal proteins of a mutant totally lacking radial spoke structures. Comparing the mutant analyses with those from wild type, we identified a group of components likely to be part of the radial spoke structure. We have already reported that two-dimensional electrophoretic analysis of the

radial spokeless mutant *pf-14* indicated that 12 polypeptides regularly present in wild type were absent from mutant axonemes and flagella. A subset of these were also lacking in the mutant *pf-1* where the axonemal protein defect could be correlated with a deficiency of radial spokeheads (2).

In the study to be reported here we have used improved procedures for analyzing axonemal proteins and have now identified 17 polypeptides as missing components in *pf-14*. Noting that some of these polypeptides showed microheterogeneity, we have demonstrated that 5 of the 17 polypeptides are phosphorylated. By applying successive extraction procedures to wild-type axonemes, we have achieved chemical dissection of the radial spoke structure. The final extraction step solubilizes the radial spokehead, leaving stalks with the residual axonemal structure. Six radial spoke polypeptides were solubilized by this procedure, and these included the five missing in the spokehead-deficient mutant *pf-1*. Analyses of the solubilized spokehead components failed to detect an associated Mg^{++} - or Ca^{++} -activated ATPase.

MATERIALS AND METHODS

Culture of cells (4), labeling with [^{35}S]sulfuric acid (7), preparation of flagella and axonemes (7), electron microscopy (7), ATPase assay (6), and estimates of molecular weight and isoelectric point of polypeptides (2), were performed as previously described.

Labeling with [^{32}P]Phosphoric Acid

Cells were grown on agar plates containing minimal I medium (3) which was further modified: phosphate concentration was lowered to 0.1 mM and 0.01 M Tris/HCl, pH 7.3, was added as buffer. Cell suspensions and subsequent washing were done in medium without phosphate. Labeling was performed in medium containing 0.15 Ci/liter of [^{32}P]phosphoric acid (specific radioactivity 30 Ci/ μ mol) at room temperature for 10 min. Cells were then sedimented by a 10-min centrifugation at 5°C in an IEC 269 rotor (Damon/IEC Div., Damon Corp., Needham Heights, Mass.) at 1,700 rpm and suspended in the solution used for deflagellation (7).

Partial Solubilization of Axonemes

In a typical experiment, ~1 mg of axonemes suspended in 450 μ l of 50 mM NaCl, 4 mM $MgCl_2$, 2.5 mM HEPES (pH 7) was mixed with 150 μ l of 2 M NaCl, 4 mM ATP, 16 mM $MgCl_2$, 4 mM dithiothreitol, and 40 mM HEPES (pH 7.2). After a 10-min incubation, the suspension was centrifuged for 40 min at 30,000 rpm in an SW65 Beckman rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The insoluble residue was suspended in 0.2 mM EDTA, 0.1% β -mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM Tris/HCl (pH 8.3) at the protein concentration of 1–2 mg/ml, dialyzed against the same solution for 2 h, and fractionated by a 30-min centrifugation at 30,000 rpm in an SW65 Beckman rotor. All operations were performed at 4°C.

Two-dimensional Electrophoresis of Polypeptides

Only two significant modifications were introduced in the procedure previously described (2). Experiments aimed at detecting the highest number of axonemal polypeptides were performed applying the samples at the anode of the (12 \times 14 \times 0.075 cm) gel containing ampholines. Then nonequilibrium pH gradient electrophoresis (10) was run for 14 h at 1.6 mA. Under these conditions all basic polypeptides remain inside the gel. The polypeptidic maps obtained by the second electrophoresis (10) were reproducible and did not show artifactual heterogeneity of basic polypeptides. This was demonstrated in separate gels where first-dimension isoelectric focusing was carried out in basic ampholines.

Analysis of Polypeptide Maps

Autoradiograms derived from paired or separate two-dimensional electrophoreses were superimposed and compared. The absence of spots or the appearance of new components were noted on each film. In this and the following paper (11) we have described only those differences that were reproducible in all maps

analyzed. The data were derived from at least three independent axoneme preparations.

RESULTS

Analysis of the radial spoke mutants, *pf-14* and *pf-1*

Our previous studies of the radial spokeless mutant *pf-14* indicated that 12 axonemal polypeptides normally found in wild type were missing in the mutant (2). The method used for isolation of axonemes has now been modified to give better preservation of axonemal morphology (7). In addition, as indicated in Materials and Methods, the procedures for two-dimensional mapping of ^{35}S -labeled axonemal polypeptides have also been modified so that basic polypeptides can be analyzed.

A comparison of Fig. 1A and B indicates that with the new procedures we could detect, in addition to the 12 polypeptides (components 1–12) originally found to be missing from *pf-14* axonemes, five newly identified polypeptides (components 13–17). Each of the 17 polypeptides has also been found to be absent from two-dimensional maps of intact flagella isolated from *pf-14*. These results suggest that their absence from *pf-14* axonemal maps is not because of selective loss during detergent solubilization of the flagellar membrane. The 17 polypeptides with their estimated molecular weights and isoelectric points are summarized in Table I. The same 17 polypeptides were found to be missing in an independently isolated allele of *pf-14*, *pf-14A*.

As seen in Fig. 1A, autoradiograms of wild-type axonemes show that several of the components missing in *pf-14* have distinctive features. Most of the *pf-14*-deficient polypeptides appear as prominent spots, yet, in comparison, components 8, 12, 13, 15, and 17 are more difficult to detect. We have also analyzed these cells by the method of silver protein staining (12), and the results are consistent with autoradiography. In addition, several of the seventeen components show microheterogeneity of charge and apparent molecular weight. This property of polypeptides 2, 3, 5, 11, 13, 16, and 17 could reflect different states of post-translational modification for these molecules.¹

The spokehead-deficient mutant *pf-1* has also been re-analyzed using the new procedures. Compared to wild type, its axonemes and flagella lack components 1, 4, 6, 9, and 10. The remainder of the 17 *pf-14* polypeptides are present.

Because axonemal polypeptides with molecular weights above 200,000 are not resolved in two-dimensional mapping procedure, these components were analyzed on appropriate one-dimensional SDS and SDS/urea gels (6). In the molecular weight range of 200,000–400,000 we have not detected any differences from wild type in the electrophoretic patterns obtained from *pf-14* and *pf-1*. Fig. 2 shows the resolution of polypeptides in the molecular weight range of 300,000–400,000 obtained with preparations of intact flagella. The major flagellar membrane protein, the high molecular weight subunits of dyneins (6), and other polypeptides resolved in this region are seen to be present in both *pf-14* and *pf-1*.

Phosphorylation of Radial Spoke Components

After our observations of microheterogeneity of some of the

¹ The microheterogeneity of polypeptides 3 and 5, which is invariably seen in original autoradiograms, is not apparent in Fig. 1A on account of reproduction at reduced magnification.

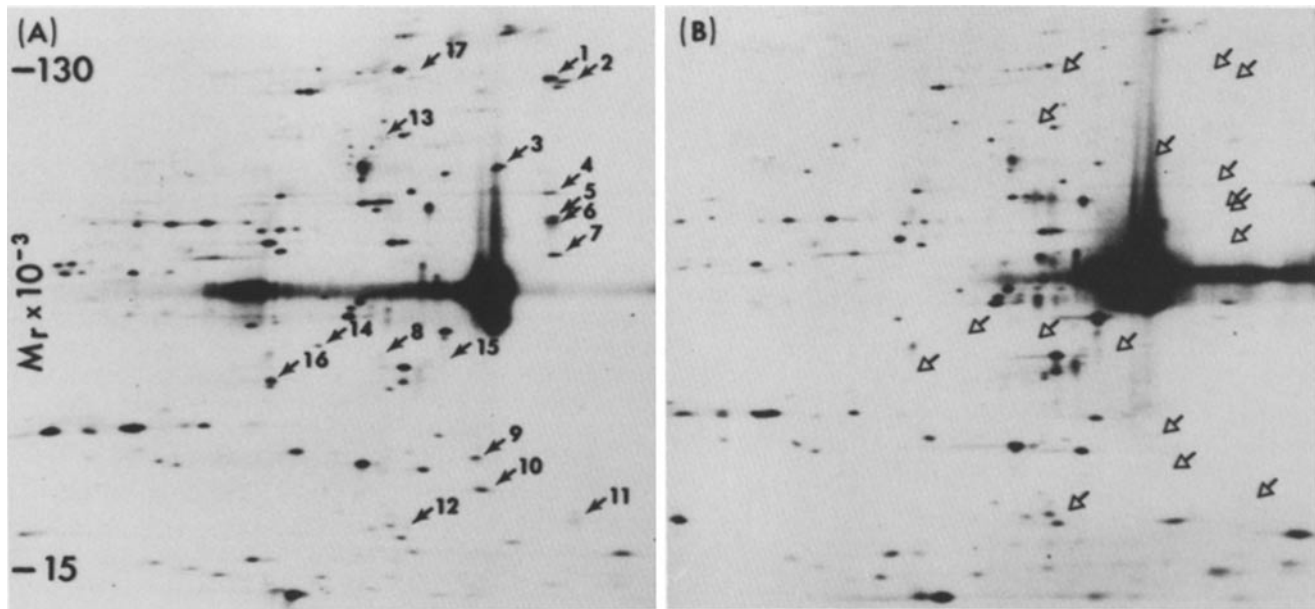


FIGURE 1 Autoradiograms of the polyacrylamide gel slabs used for the two-dimensional separation of ^{35}S -labeled axonemal polypeptides. The relevant portions of the original maps are shown. Proteins were applied at the anode. Basic polypeptides are on the left side. Tubulin subunits are not resolved and form a streak as a consequence of overloading. (A) Wild-type axonemes. Arrows point to 17 polypeptides that are absent in the map shown in panel B. 12 polypeptides previously characterized (2) are numbered in order of decreasing molecular weight; components newly identified are indicated with progressive numbers. (B) *pf-14* axonemes. Arrows with open arrowheads indicate the positions where polypeptides are missing.

TABLE I
Polypeptides Missing from *pf-14* Flagella

Polypeptide No.	mol wt	Isoelectric point
1	123,000	5.2
2	118,000	5.0
3	86,000	5.5
4	76,000	5.1
5	69,000	5.1
6	67,000	5.1
7	58,000	5.1
8	40,000	6.5
9	26,000	5.7
10	24,000	5.6
11	22,000	4.8
12	20,000	6.3
13	98,000	6.3
14	41,000	6.8
15	38,000	5.7
16	34,000	7.1
17	124,000	6.2

radial spoke polypeptides, we studied protein phosphorylation by carrying out short in vivo ^{32}P pulse labeling as described in Materials and Methods. The results were analyzed by two-dimensional electrophoretic analysis of axonemal proteins.

Fig. 3A shows a representative map with >80 ^{32}P -labeled components resolved in the molecular weight range 25,000–130,000. Matching this kind of map with those obtained by ^{35}S labeling indicated that radial spoke components 2, 3, 5, 13, and 17 contained ^{32}P . Component 2 was labeled to a higher extent than all other spoke polypeptides and appeared to be formed by a family of polypeptides with different isoelectric points and molecular weights. Tubulin subunits, which appear as major spots in the ^{35}S label maps, were not pulse labeled by ^{32}P .

The identification of phosphorylated radial spoke compo-

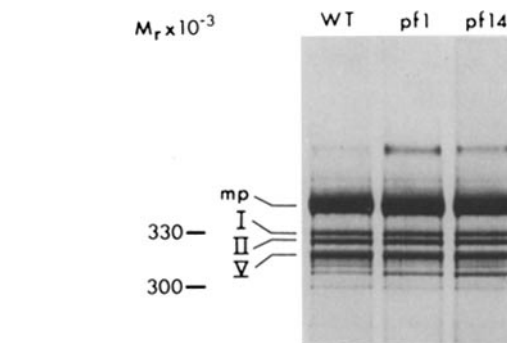


FIGURE 2 Autoradiogram of the polyacrylamide gel slab used for the resolution of flagellar polypeptides of molecular weight 300,000–400,000. Only a portion of the original electrophoretogram is shown. Equal amounts of radioactive proteins from the strains indicated above each lane were applied to the gel. *mp* Indicates a membrane polypeptide and roman numbers the major polypeptides associated with the arm structures (6, 7).

nents could be confirmed by experiments performed with ^{32}P -labeled *pf-14* and *pf-1* axonemal polypeptides. As shown in Fig. 3B, components 2, 3, 5, 13, and 17 are missing from ^{32}P maps of *pf-14*, as would be expected based on the deficiency of these polypeptides in the spokeless mutant. In *pf-1*, however, (data not shown) where the proteins are known to be present the ^{32}P maps resemble those of Fig. 3A. It should be noted in Fig. 3B that the ^{32}P labeling of other axonemal polypeptides was not affected by the absence of radial spokes.

To determine whether phosphorylation was a feature of the formation of new flagella we carried out pulse ^{32}P labeling in the presence of colchicine. At a concentration of colchicine, 3 mg/ml, sufficient to inhibit flagellar regeneration (13), no alteration in the polypeptide maps was found.

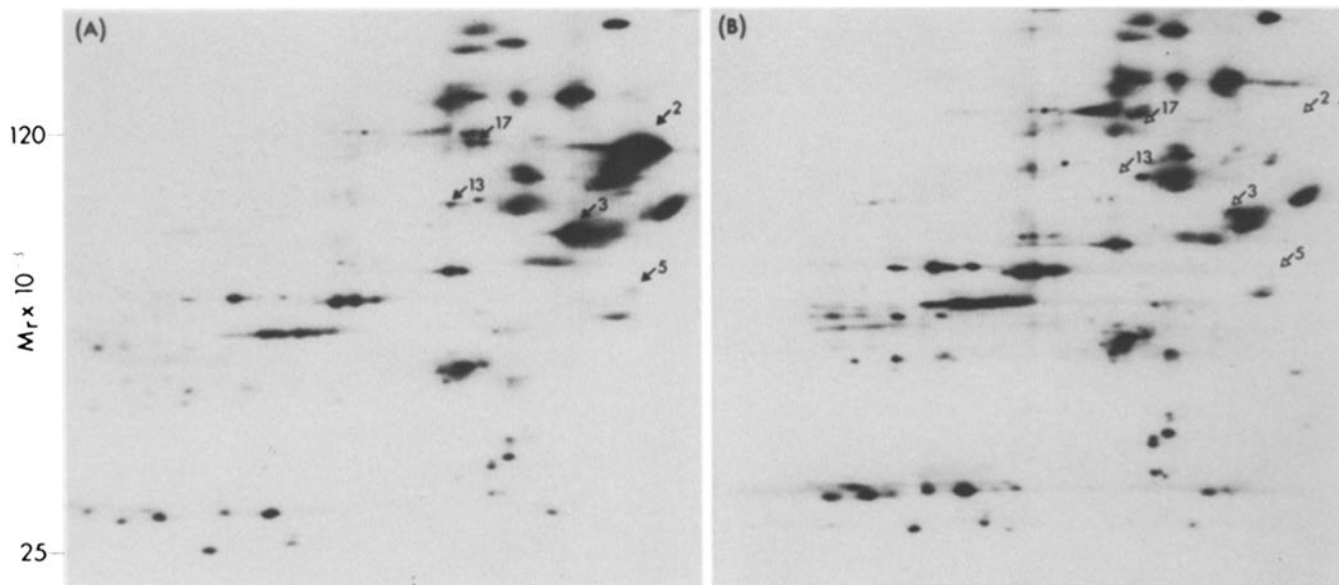


FIGURE 3 Portions of autoradiograms of polyacrylamide gel slabs as in Fig. 1. Pulse labeling was obtained with [32 P]phosphoric acid. (A) Wild-type axonemal polypeptides. Arrows point to five polypeptides of the group of seventeen that are missing in *pf-14*. (B) *pf-14* axonemal polypeptides. Arrows with empty arrowheads indicate the positions where 32 P-labeled polypeptides should be detected.

Chemical Dissociation of the Radial Spokehead from the Stalk

We have utilized chemical dissection procedures on wild-type axonemes to confirm the results, obtained from our analysis of the radial spokeheadless mutant *pf-1*, that components 1, 4, 6, 9, and 10 were localized in the spokehead. This approach also provided a soluble preparation with which to study possible enzymatic activity of spokehead polypeptides.

As starting material for the fractionation, wild-type axonemes pre-extracted with 0.5 M NaCl were used. As we have previously shown (6), brief high salt treatment solubilizes nearly completely the dynein heavy subunits and 35% of the axonemal protein mass, but leaves the radial spokes structurally intact. The retention of spokes in high salt extracted axonemes is illustrated in Fig. 4A. Fig. 5A shows that the 17 polypeptides missing in *pf-14* axonemes are present in the 0.5-M NaCl-insoluble residue.

A short dialysis against a low ionic strength solution, as described in Materials and Methods, preferentially solubilized a small group of the residual axonemal components. Fig. 5B and C show that of the 17 components in question only 1, 4, 5, 6, 9, and 10 were extracted during the dialysis; the remaining 11 components were found in the insoluble residue. The separation of the polypeptides is nearly complete because very little cross-contamination is detectable in the two maps.

Fig. 4B and C illustrate the morphology of the low ionic strength extracted axonemes. In longitudinal sections of extracted axonemes (for example, see Fig. 4B), radial spoke structures of varying lengths are seen to extend from the outer doublet microtubules. Morphologically distinct radial spokeheads are absent. Except for the apparent loss of the radial spokeheads, the morphology of axonemes in the low ionic strength-insoluble residue is very similar to the initial high salt-extracted axonemes (compare Fig. 4C with Fig. 3 in reference 6).

It should be noted that the results obtained by chemical

dissection of the spokehead from the stalk shows one difference from mutant analysis. Component 5, which is present in *pf-1*, is selectively solubilized with the mutant-deficient polypeptides 1, 4, 6, 9, and 10.

Association of ATPase Activity with Radial Spokehead Components

It has been suggested, based on histochemical experiments (14), that an ATPase might be associated with radial spokes. The chemical dissection procedure just described provided appropriate material to test this possibility. The first step, salt extraction, solubilizes nearly completely the substantial activities of dynein ATPases of outer and inner arms (6) leaving radial spokes with the insoluble residue. The subsequent extraction by dialysis against low ionic strength buffers solubilizes completely the spokehead and polypeptide components 1, 4, 5, 6, 9, and 10 in a preparation that is suitable for testing ATPase activity. To separate ATPase activity that might be associated with these components from activity contributed by residual dyneins, the analysis was refined by fractionating the low ionic strength extract by sedimentation on a 5–20% sucrose gradient. Fig. 6A shows the distribution of protein mass after sedimentation. Fractions were analyzed by two-dimensional electrophoresis to localize spokehead components. The polypeptide map of fraction 36 is shown in Fig. 6B. It is to be noted that tubulins and spokehead components comprise the major polypeptides of this map. The same components are present in fractions 30–40. Assays for Mg^{++} - or Ca^{++} -dependent ATPase were carried out on fractions 30–41. As shown in Fig. 6A, such activities were negligible in the region of interest.

DISCUSSION

Through the use of one- and two-dimensional electrophoretic techniques which have the power to resolve >170 axonemal polypeptides, axonemes of a mutant lacking radial spokes were found to be deficient in 17 polypeptides regularly present in

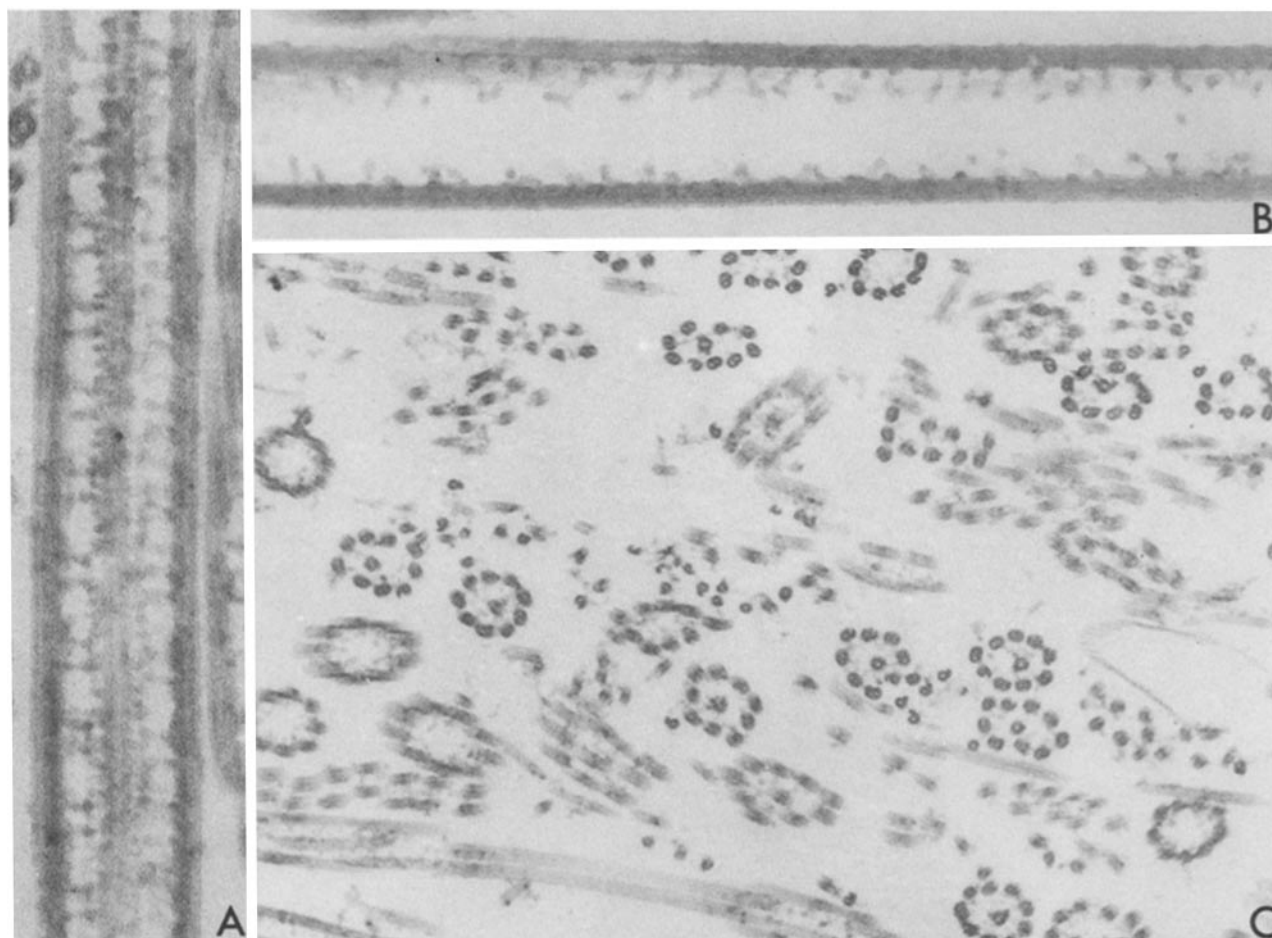


FIGURE 4 Thin-section electron micrographs illustrating the effects of sequential extraction procedures on the morphology of wild-type axonemes. (A) A representative longitudinal section of an axoneme extracted with 0.5 M NaCl. In the high salt-extracted axoneme, a single row of radial spokes with normal periodic frequency is seen to extend from the outer doublet at the left toward the central tubules. Visible at the bottom left are pairs of radial spokes in which the proximal stalks terminate in distinct spokeheads. $\times 94,000$. (B and C) Micrographs of the residue pellet after dialysis of high salt-treated axonemes to a low ionic strength solution. In the longitudinal image of an extracted axoneme, radial spoke structures of variable lengths and frequencies are seen, but morphologically distinct spokeheads are absent. Except for the loss of the radial spokeheads, the morphology of the axonemes in the low ionic strength-insoluble residue is very similar to the initial high salt-extracted axonemes (compare Fig. 4 C with Fig. 3 in reference 6). (B) $\times 94,000$. (C) $\times 42,000$.

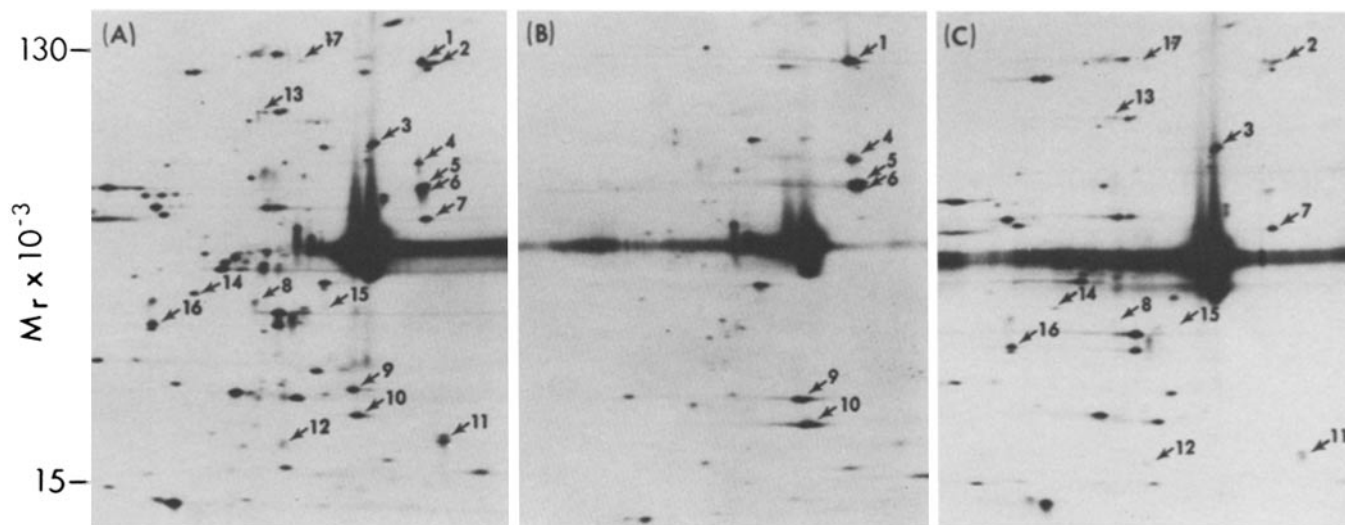


FIGURE 5 Portions of autoradiograms of polyacrylamide gel slabs. Same conditions as in Fig. 1. Arrows and numbers identify the polypeptides that are putative components of radial spokes and are enriched in the different fractions. (A) Polypeptides from axonemes that have been extracted with 0.5 M NaCl. (B) Polypeptides solubilized from salt-extracted axonemes by dialysis against a solution of low ionic strength. (C) Polypeptides present in the insoluble residue after the dialysis.

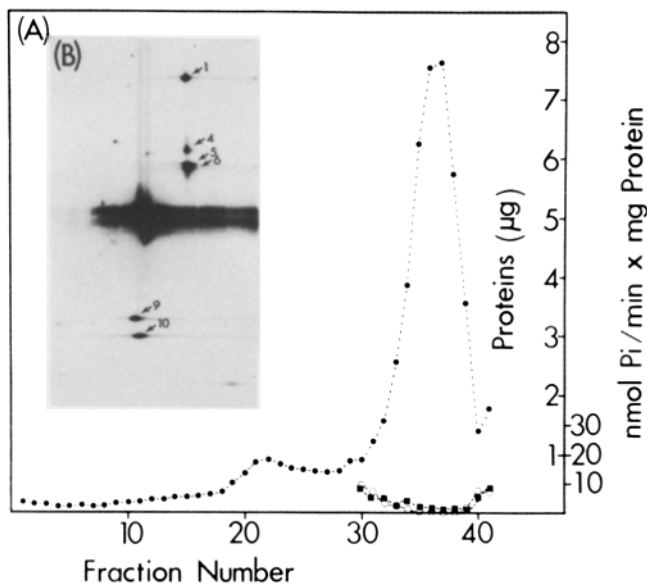


FIGURE 6 (A) Sedimentation pattern of the low ionic strength extract prepared as described in Materials and Methods. Sample was loaded on a 4 ml 5–20% sucrose gradient in 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM Tris/HCl (pH 8.3). Centrifugation was done in an IEC SB405 rotor at 42,000 rpm for 10 h at 5°C. Top of the gradient is on the right side. (B) Autoradiogram of the polyacrylamide gel used for the two-dimensional separation of polypeptides present in fraction 36 of the sucrose gradient. Same conditions as in Fig. 1. Arrows and numbers point to the spokehead polypeptides.

wild type. It is likely that most or all of this group are components of radial spoke structures. The same components are missing in flagellar preparations. Mutants showing defects in other axonemal structures, i.e., central microtubule complex (15), and inner and outer dynein arms (7) do not show alterations in these 17 polypeptides, while other mutants affecting the spoke system do not show missing polypeptides outside of this group (11). Extractive procedures applied to wild-type axonemes which solubilize dynein arms along with ~35% of the axonemal protein mass, preserve doublet and radial spoke structures along with the 17 polypeptides in question.

It is also likely, based on analysis of the mutant *pf-1* that a subset of this group, components 1, 4, 6, 9, and 10, are localized in the radial spokehead. This assignment is supported also by the extractive experiments in which the same subset of polypeptides is rendered soluble under conditions which result in loss of radial spokeheads from axonemes already stripped of dynein arms by salt extraction. The secondary extraction procedure also solubilized component 5, which suggests that it may serve as a link between spokehead and stalk. By elimination then, the remaining components (2, 3, 7, 8, 11–17) can be assigned to the proximal radial spoke stalk.

As resolved by two-dimensional electrophoresis, the 17 radial spoke components are present at different concentrations in the gel slabs. Therefore, assuming that there is no preferential loss of molecules in any step of the procedure, the polypeptides appear to be assembled in the structure at different molar ratios. It is possible that the minor components are assembled in substructures, like the thin filaments observed between the spokeheads, or in a subset of radial spokes having different molecular composition.

It has been reported recently that electrophoretic analysis of

flagellar preparations from *pf-1* and *pf-14* showed retardation of mobility of the major membrane polypeptide compared with wild type; in some mutant preparations normal and abnormal polypeptides were found and in some wild-type preparations a small amount of the abnormal species was present (16). We have not detected such alterations in flagellar preparations of *pf-1*, *pf-14*, or wild type using electrophoretic conditions (see Fig. 2) which resolve clearly the major membrane polypeptide from the large subunits of dynein, and the dynein subunits from each other (6). In addition, as will be described in the following paper, no such alteration was found in newly identified radial spoke mutants representing five independent genetic loci (11).

It has been proposed that the interaction between the radial spokes and the projections from the central pair structures may be mediated by a dynein-like ATPase (14, 17). However, it seems unlikely that a dynein ATPase is associated with radial spokes. Analysis of the radial spokeless mutant *pf-14* reveals no deficiencies in the molecular weight range above 300,000, the size range for the large subunits of dyneins. Furthermore, under conditions in which radial spokehead components can be solubilized and analyzed in isolation from the overwhelming activity of arm-associated dyneins, no associated ATPase activity has been found. It is possible, of course, that enzymatic activity is lost in the course of the extraction procedure.

The significance of incorporation of [³²P]phosphate into a subset of the radial spoke proteins during short pulses cannot yet be fully assessed. It is of interest that the phosphorylated polypeptides seem to be stalk components, i.e., 2, 3, 13, and 17, or in the case of component 5, a link between stalk and spokehead. Furthermore, each of these polypeptides shows charge heterogeneity in two-dimensional maps derived from axonemes labeled for long periods with ³⁵SO₄, suggesting that the components may exist in variable states of phosphorylation. As the present phosphorylation studies are not quantitative, it is uncertain whether it is more likely that a phosphorylation cycle is a feature of the flagellar beat cycle, or whether it is associated with radial spoke assembly. The fact that the ³²P pulse labeling pattern is not altered in the paralyzed mutants *pf-1* (deficient in radial spokeheads) favors the second possibility. Although colchicine at a concentration adequate to block flagellar regeneration does not alter the ³²P pulse pattern, it is not known what effect this drug might have on turnover or remodeling processes.

The 17 components shown to be associated with radial spokes have provided a signature for detecting other flagellar mutants. As will be shown in the subsequent paper, mutants representing six independent loci in addition to *pf-14* have been identified. Among the new mutants is a regulatory mutant showing specific defects in radial spoke phosphorylation.

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