

EFFECT OF SOLUTION COMPOSITION AND PROTEOLYSIS ON THE CONFORMATION OF AXONEMAL COMPONENTS

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ABSTRACT

The effect of solution composition and enzymic proteolysis on axonemes prepared from the sperm of sea urchins, *Tripneustes gratilla*, has been investigated. Aliquots of axonemes, prepared by treatment of sperm with Triton X-100 and differential centrifugation, were transferred to solutions of different composition with and without intervening tryptic proteolysis, and the particle conformations observed by dark-field and electron microscopy. In most solutions particles in partially digested preparations underwent conformational transformations to coiled or helix-like forms. Proteolysis was accompanied by an increase in the ATPase activity of the digest: by centrifuging down the insoluble digestion products it was shown that digestion resulted in the appearance of ATPase activity in the soluble phase with a concomitant decrease in ATPase activity in the pellet fraction. Gel electrophoresis showed this corresponded to the appearance of dynein in the supernatant and a decrease in dynein associated with the insoluble fraction. Supernatant dynein had a greater specific ATPase activity than dynein extracted from axonemes. Observations on specimens prepared for electron microscopy by thin sectioning allowed a rough correlation to be made between the dark-field observations, chemical analyses, and morphological alterations attendant with the proteolysis and solution conditions. It is concluded that in the intact axoneme the doublet tubules are under considerable tension and that proteolytic destruction of physical restraining elements allows spontaneous conformational alterations of the digestion products. In addition, proteolysis increases the specific ATPase activity of dynein and removes a portion of it from the axonemal structure.

INTRODUCTION

Ciliary and flagellar motion is characterized by the passage of a bending moment along the length of the motile organelle. Cinematographs of swimming ciliates or flagellates (Rikmenspoel and van Herpen, 1956) are very striking demonstrations of this attribute: somewhat less forceful demonstrations are sequential dark- or bright-field micrographs made using stroboscopic techniques

(Brokaw, 1965; Gray, 1955). Such observations suggest that the axoneme is a very flexible structure that allows extensive relative displacement of its constituent parts. Freedom of motion between the component doublet tubules of the axoneme is also an inherent feature of current models of ciliary or flagellar motion (Brokaw, 1968, 1971; Rikmenspoel, 1971, Satir, 1968; Sleight, 1968).

However, observations on isolated axonemes and even their component structures, the doublet tubules and central pair of tubules, are not entirely in accord with these expectations. Optical and electron microscope observation of these elements are generally suggestive of fairly rigid structures. Occasionally gentle curvature is noted or sharp bends suggestive of preparative damage, particularly in electron microscope specimens, but otherwise they are fairly straight (Behnke and Zelander, 1967; Brokaw, 1968; Burton, 1966 *a*; Henley et al., 1969; Jensen and Bajer, 1969; Porter, 1966).

Consideration of the ultrastructure of the axoneme provides ample evidence suggestive of structural resistance to relative displacement among the component parts. Most notably the nine doublet tubules are circumferentially linked by the protein nexin and radially cross linked to the sheath of the central pair by the radial spokes (Fawcett, 1962; Gibbons, 1963; Stephens, 1970 *b*). In addition each A tubule is reinforced with two rows of dynein that may make contact with the adjacent B tubules (Gibbons and Rowe, 1965). Greater flexibility would be expected for the individual doublet tubules, but in common with other cytoplasmic microtubular structures they too usually appear fairly straight—implying rigidity.

When the integrity of the axonemal apparatus has been partially destroyed by brief treatment with trypsin, a tendency for the subunit structures to undergo conformational alteration has been noted (Summers and Gibbons, 1971). In the presence of ATP the doublet tubules move relative to one another and in addition they occasionally assume a coiled configuration. This implies that not only do the various cross-linking elements in the axonemal structure restrict relative displacement between the tubular elements but they also prevent the latter elements from assuming more favorable conformations possibly induced by environmental conditions.

The current conception of the ultrastructure of the doublet tubule is that the A tubule consists of 13 longitudinally arranged protofilaments, a number of which (three to five) form a common wall with the B tubule that has an additional 10 (Ringo, 1967). The protofilaments are believed to be constituted of periodically arranged globular units. Although the composition of the subunits of microtubules from a variety of sources has not been shown to be identical, there is evidence for considerable homology (Arnott and Smith, 1969;

Burton, 1966 *b*, 1970; Hooke et al., 1967). Nevertheless, within the microtubules the substructural arrangement of the subunits might be expected to vary in tubules from different sources that might perform different functions (Behnke and Forer, 1967). Similarly it might be expected that the subunit arrangement would be influenced by composition of the solution and in turn would lead to an alteration of the overall conformation of the tubules (Henley, 1970). Burton (1968), Thomas (1970), and Thomas and Henley (1971) have reported that in some microtubules the subunits may be arranged in either a helical or protofibrillar configuration and that environmental conditions may have some influence on which arrangement occurs. Whether such polymorphism is related to higher order conformational alterations of the structures is not known (Costello and Henley, 1969).

The effect of solution composition on the gross conformation of axonemes and fragments that result from tryptic digestion has been explored. It has been found that once the integrity of the axoneme has been disrupted the resulting fragments frequently assume helical conformations in a variety of solutions. Soluble proteinaceous material displaying ATPase activity is released during the course of digestion and, in fact, the activity of this material (ostensibly dynein) is enhanced by the digestion process.

MATERIALS AND METHODS

Preparation of Axonemes

Axonemes were prepared from sperm (obtained by extrusion from *Tripneustes gratilla* after injection with 0.56 M KCl) by several treatments with Triton X-100 to remove membranous material and repeated differential centrifugation to remove heavy contaminants, principally heads. The procedures and solutions used were essentially as described by Gibbons and Fronk (1972) and Gibbons and Gibbons (1972). The final preparation of purified axonemes was usually somewhat less than 5% of the original sperm protein as determined by the Lowry technique.

The ATPase activity of freshly prepared axonemes was found to be 0.28 $\mu\text{M P}_i/\text{mg per min}$ with a range of 0.16–0.41 $\mu\text{M P}_i/\text{mg per min}$ for 20 preparations. Outer fibers (OF's) were prepared by removal of the dynein arms from axonemes either by extraction with 0.5 or 0.6 M KCl or by dialysis vs. 2 mM Tris-HCl, pH 8 (containing 10^{-4} M EDTA and 1 mM dithiothreitol [DTT]). When tested for ATPase activity the OF's prepared by extraction with KCl

were found to have an activity of ca. $0.08 \mu\text{M P}_i/\text{mg per min}$ (seven preparations). The OF's prepared by dialysis against low ionic strength buffers had a more variable activity, occasionally being as much as $0.1 \mu\text{M P}_i/\text{mg per min}$ but usually closer to zero (e.g., $0.03 \mu\text{M P}_i/\text{mg per min}$ for five preparations). For use in digestion experiments axonemes and OF's were resuspended in suspending medium (SM) (25 mM Tris-HCl, 2.5 mM MgSO_4 , 0.25 mM EDTA, 0.1 mM DTT, pH 8). It might be noted that in contrast to axonemes OF's were rather difficult to resuspend and frequently had to be homogenized to break up aggregates. Thus the OF's observed by dark-field microscopy were, on the average, shorter and more prone to internal damage than axonemes.

Tryptic Digestion of Axonemes and OF's

Tryptic digestion of either axonemes or OF's suspended in SM was carried out by adding aliquots of trypsin in H_2O (pH 8, 25°C), the extent of digestion being followed by observing the decrease in turbidity at 350 nm. Since the rate of decrease in turbidity was influenced by agitation of the solution, controlled conditions were used. Proteolysis was terminated by addition of excess soybean trypsin inhibitor and immediate transferral of the solution to an ice bath. Standard assays of trypsin activity with α -N-benzoyl-L-arginine ethyl ester (BAEE, Sigma Chemical Co., St. Louis, Mo.) showed it to be within about 20% of the manufacturer's stated values.

Preparation of Doublet Tubules, A Tubules, and B Tubulin

Summers and Gibbons, 1971, have shown that ATP causes disintegration of axonemes that have been briefly treated with trypsin. Alternatively the same result can be achieved by digestion of axonemes in the presence of ATP. Centrifugation of such solutions for 15 min at 12,000 *g* was carried out to remove both any remaining undigested axonemes as well as many partially digested ones that had failed to disintegrate. After such centrifugation, the supernatant fraction contained doublet tubules as verified by observations on electron microscope specimens prepared by negative staining (see Fig. 2).

A tubules were prepared from OF's essentially by the thermal fractionation procedure of Stephens (1970 *a*); however, higher temperatures (50°C) and longer heating periods (ca. 10 min) were required to dissociate the B tubule. Electron microscope examination of negatively stained specimens of such preparations revealed the presence of singlet tubules (see Fig. 3). The supernatant fraction had an OD of 14 (at 280 nm) for a 1% solution. No filaments were found in electron microscope specimens of this material.

pH-Stat Assays

ATPase activities of the various axonemal preparations and dynein were determined at pH 8.0, 25°C with a Sargent recording pH-stat. 2 mM NaOH was used as a titrant. The standard assay solution was 4 mM MgSO_4 , 0.5 mM EDTA, and 0.1 M KCl. The ATP concentration was about 0.5 mM, although occasionally other concentrations were used in specific experiments. Details of the procedure have been described previously (Gibbons and Fronk, 1972; Gibbons and Gibbons, 1972).

Observation of Preparations by Light- and Electron Microscopy

Dark-field observations of axonemal preparations or digests were made using a Zeiss research microscope on aliquots diluted to a concentration of about 10^{-3} mg/ml with the solution of choice. Photomicrographs were generally made at a magnification of 640 on Kodak 2475 recording film that was developed in HC-110 developer.

Specimens negatively stained with uranyl acetate were observed in a Philips EM 300 and micrographs were usually made at an electron optical magnification of 56,000.

Some specimens were prepared by the critical point procedure of Anderson (1951) and subsequently viewed and photographed in an Hitachi HU 11C electron microscope.

Gel Electrophoresis

Gel electrophoresis of a number of specimens was carried out essentially by the procedure of Weber and Osborn (1969). Before electrophoresis samples were dialyzed for 2 or more h at ca. 38°C vs. a 20-fold excess of solution containing 1% sodium lauryl sulfate, 1% β -mercaptoethanol, 7 M urea, and 10 mM phosphate buffer, pH 7. After 3 h of electrophoresis on 8% acrylamide gels, the gels were stained for an hour or more with 0.5% Buffalo Black in 7% acetic acid. Destaining was carried out electrophoretically.

Preparation of Thin Sections for

Electron Microscopy

Fixation of pelleted axonemal digests was accomplished with 2% glutaraldehyde buffered to pH 8.2 with phosphate. After rinsing and postfixation with 2% OsO_4 , the pellets were embedded in Araldite and sectioned for microscopy (details of the preparative procedure are given by Gibbons and Gibbons, 1972).

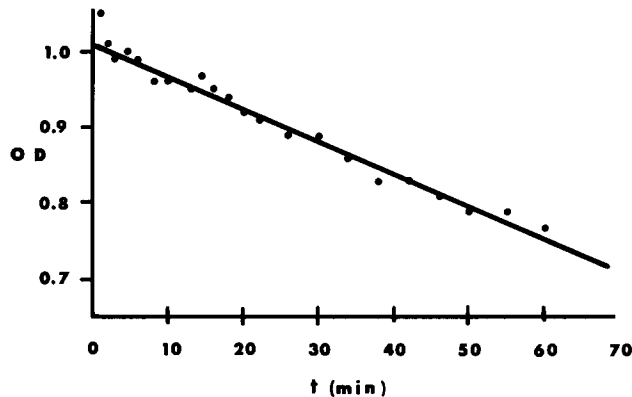


FIGURE 1 Variation of optical density with time of digestion for axonemes. Axonemes in suspending media at pH 8 were digested with 5×10^{-4} mg trypsin.

RESULTS

Digestion of Axonemes and OF's

Since the turbidity of solutions of axonemes (or OF's) decreases as digestion proceeds, the relative optical density (measured at 350 nm) at any time may be used as a measure of the extent of digestion. The rate and extent of decrease in turbidity depends on at least four factors: agitation, purity of the substrate preparation, substrate/enzyme ratio, and composition of the suspension medium. By utilizing a consistent agitation procedure and monitoring the axonemal preparations to ensure that contamination with sperm heads was minimized, errors due to these factors could be essentially eliminated. The decrease in turbidity (from $OD_{t=0}$ to $\frac{1}{2} OD_{t=0}$) was found to be approximately proportional to the substrate/enzyme ratio (over the range, 5,000/1–500/1). Addition of ATP to the reaction mixture either before or during proteolysis led to an increase in the rate of decrease in turbidity for digestion of axonemes but not OF's.

Although in some instances the decrease in turbidity did not terminate with the addition of inhibitor, control experiments were unsuccessful in providing an explanation for this observation. Other experiments on the pH-stat did show that addition of inhibitor always led to cessation of H^+ ion production within 1 or 2 min.

By withdrawing aliquots from digestion mixtures at varying intervals of time and adding them to inhibitor, a series of axonemal samples that had been digested to different extents was obtained. From these initial aliquots containing par-

tially digested axonemes, trypsin, and inhibitor, further dilutions were made into other solvents to determine the effects of various agents on the conformation of the axonemes (or OF's) that had been digested to different extents. Data for a typical experiment are given in Fig. 1. 14 ml of axonemes were digested (on a magnetic stirrer) at pH 8, in SM with 5×10^{-4} mg of trypsin. At various intervals $\frac{1}{2}$ ml aliquots were withdrawn from the reaction mixture and added to 1 ml of inhibitor (2×10^{-3} mg/ml). From this series of aliquots three more series were made by dilution. One contained 10^{-4} M ATP, the second contained 10^{-4} M ATP and 2% glutaraldehyde in 25 mM PO_4 , pH 8, and the third was dialyzed vs. 2 mM Tris (containing 10^{-4} M EDTA and 10^{-4} M DTT), pH 8.

Each of the aliquots prepared in this way was examined by dark-field microscopy (described in the next section). Since the results of these observations showed that the conformations of the digestion products were not very sensitively dependent on the extent of digestion, in subsequent experiments many fewer aliquots were prepared. Furthermore, digestion was allowed to proceed to a greater extent.

Microscope Observations

When observed by dark-field microscopy, preparations of axonemes (Fig. 5) or OF's (Fig. 6) are seen to contain large numbers of relatively straight, elongate particles. (The term "particle" will be applied to any of the elongate objects viewed by dark-field or electron microscopy regardless of their conformation. Thus it may

apply to OF's, axonemes, fragments of either, groups of doublet tubules, or even individual doublet tubules. Many preparations no doubt contain several different types of such particles. Somewhat more precise characterization of the particles observed in digestion mixtures will be denoted by the terms "fragment" or "disintegration product" as defined below.)

Limited digests of axonemes, to which nothing other than inhibitor has been added, have much the same appearance as the original axonemal preparation as long as the turbidity has not decreased to less than 80% of the initial value (i.e., $OD_{i^{350}} \geq 80\% OD_{i^{350}}$). After digestion exceeds about 70% $OD_{i^{350}}$ a very significant decrease in the proportion of axonemes or fragments is observed, suggesting extensive disintegration of the partially digested axonemes. For convenience in further discussion, the elongate particles whose scattering intensity is similar to that of undigested axonemes or OF's and that usually exhibit the least tendency toward curvature, will be referred to as axonemes, OF's, or fragments of either. Thus, the term "fragment" will be used to describe elongate particles produced from axonemes (or OF's) by primarily transverse cleavage. In actual fact various components may have been removed from the original structure so that a fragment is really only a group of nine or slightly fewer doublet tubules. In favorable instances the number of doublet tubules in a group may be estimated from the relative intensity of the scattered radiation (see, e.g., Fig. 9 *b, e*) but in general such estimates are not possible and it would seem preferable to accept less specific characterization of the particles observed. With more extended digestion (to about 50% $OD_{i^{350}}$) the particles observed had very low scattering intensities, were noticeably shorter than in undigested samples and frequently had distorted, irregular conformations. No doubt such particles are produced by transverse cleavage of axonemes followed by separation of the structures into small groups of doublet tubules or in many cases individual doublet tubules. Such individual doublet tubules or small groups of doublet tubules will hereafter be designated disintegration products (DP's) to distinguish them from the larger groupings of doublet tubules previously denoted fragments.

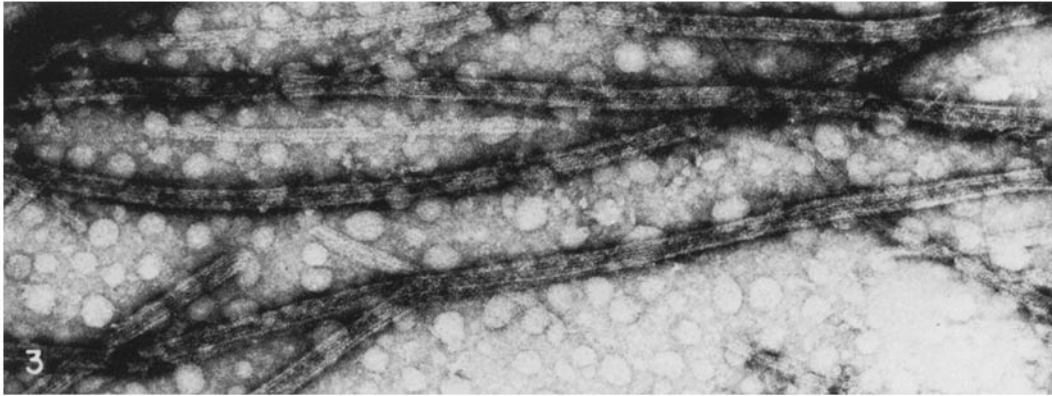
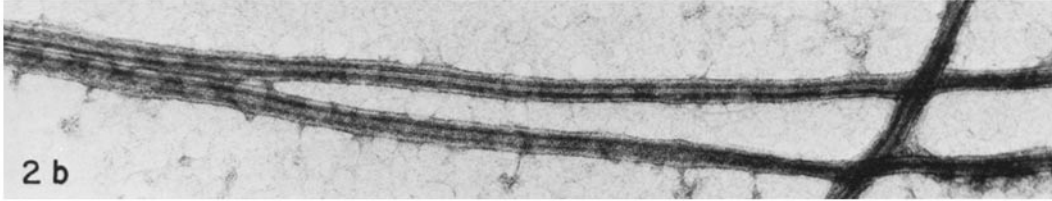
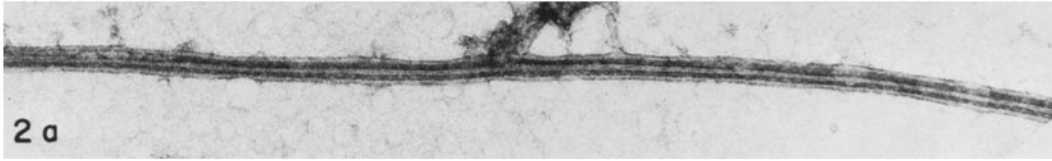
In digests to which ATP had been added extensive disintegration of axonemes occurred in much less digested preparations. Thus, in preparations

whose turbidity was 80% $OD_{i^{350}}$, virtually no axonemes or long fragments were observed after the addition of ATP. This observation can be correlated with a simultaneous decrease in the turbidity of the suspension when the ATP is added (for further discussion of this point see preceding results section and Summers and Gibbons, 1973).

Dialysis of axonemes against low ionic strength buffers results in extraction of dynein and morphologically in the disappearance of arms from the doublet tubules (Gibbons, 1965 *b*, 1967). Dark-field observations revealed that after such extraction axonemes tended to be gently curved, rather than straight as in unextracted preparations. Observations on axonemes that were dialyzed after treatment with trypsin showed that the resultant groups of tubules were also curved and that the less intensely scattering digestion products (DP's) were frequently coiled; whereas, longer more intensely scattering particles (fragments) were just curved. The greater the extent of digestion the larger the fraction of coiled particles that were observed.

The appearance of preparations of OF's (see Fig. 6) and digests of OF's was much the same as for axonemes and their digests; however, the average length of the OF's was significantly less than for axonemes, no doubt due to breakage during the additional preparative manipulations. In digests of OF's considerably more disintegration of the OF's seemed to have occurred at any given level of digestion when compared to axonemes digested to the same extent. Further support for these observations was provided by direct comparison of a partial axonemal digest (90% $OD_{i^{350}}$) with an aliquot of the same digest from which the dynein had been extracted with 0.6 M KCl. The control aliquot contained a significantly larger fraction of axonemes and fragments than the extracted fraction. Addition of ATP to OF's or their digests had no observable effect on the particle size or apparent extent of digestion, nor was there any significant decrease in the turbidity. OF's that had been dialyzed against low ionic strength buffers showed relatively the same conformational alterations as axonemes (i.e., tendency toward curvature), examples of which are shown in Figs. 11 *a-d*.

Addition of glutaraldehyde to any of the *digests* led to a very surprising alteration in the appearance of the fragments and DP's, many of them



assuming an apparently helical conformation (see Figs. 9 *a-e*). Although the regularity of many of the helices is quite striking, it is obvious that the helical parameters are not the same for all the particles, thereby, precluding definitive classification. Distinctive coils were most clearly observed in digests dialyzed or diluted into a low ionic strength buffer (Fig. 11). The helical parameters do not vary in any way that could be systematically correlated with variations in extent of digestion, which seemed to be more clearly related to the proportion of particles that assumed a helical conformation. In digests of axonemes to less than about 95% OD_i^{350} few, if any, helices were observed. Noticeable helices were evident in digests to 90% OD_i^{350} and if digestion was allowed to proceed to 80-70% OD_i^{350} the major portion of the observed digestion products had helical conformations. This was still essentially true for digestion to OD_i^{350} 's less than 70% OD_i^{350} , but fewer particles were usually observed. Basically the same observations were made on digests of OF's but in correspondingly less digested specimens.

In view of the rather striking effect of glutaraldehyde on the conformation of particles in axonemal and OF digests, the relationships, if any, between extent of digestion, solution composition, and particle conformation were explored further. Observations were made on axonemal (or OF) preparations that had been digested to 90%, 70%, and 50% of their initial turbidity and transferred to a number of solvents.

In several of the solvent systems, particles having conformations that could be described as helices, (Figs. 9, 10, 13, 14, 16), helical-coils (Figs. 12, 15), or coils were observed (Fig. 11). These are tabulated in Table I. Although the very qualitative nature of the observations prohibits precise categorization, it is clear that in these solutions, as in glutaraldehyde discussed previously, increasing digestion results in a larger proportion of particles with the helical-coil type conformation, with the

perfection of the helices being somewhat dependent on the composition of the solution. For a number of reagents (e.g., phosphotungstic acid (PTA), Sarkosyl, colchicine) the observations seem to result from the dissociating effect of the reagent on the axonemes and their digestion products, higher concentrations causing an earlier disappearance of large groups of tubules. In SM many of the particles are twisted or misshapen but they have not assumed a conformation that could reasonably be described as a coil or helix. Urea (0.5 and 2 M), guanidine-hydrochloride (0.5 and 2 M), and ethanol (20%) caused complete dissolution of axonemes, OF's and their digestion products. Possibly at lower concentrations observation of some particles might occur. Random aggregation of axonemes, OF's, and their fragments was noted in uranyl acetate, glutaraldehyde, formaldehyde, and in acid.

These observations make clear that the solution composition has a significant influence on the conformation of axonemes or OF's that have been digested with trypsin and that the extent of influence is dependent upon the extent of digestion. Note, that undigested axonemes or OF's did not tend to undergo these conformational alterations when put in glutaraldehyde (Figs. 7 and 8).

Other solution conditions were explored somewhat more systematically as summarized below:

EFFECT OF pH: At pH's <4 partially digested axonemes or OF's aggregated, but as the pH was raised fragments and coiled particles were observed. Increasing pH was accompanied by apparently increasing dissociation of digestion products until at pH's > 10 complete dissolution occurred.

EFFECT OF IONIC STRENGTH: Low ionic strength enhanced dissociation of partially digested axonemes and OF's and the formation of helical coils. At higher ionic strengths dissociation was not as evident but helix formation of digestion products was not entirely prevented.

FIGURE 2 Doublet tubules negatively stained with uranyl acetate. Tubules were prepared from axonemes by brief treatment with trypsin and ATP followed by centrifugation to remove debris. (See text for details.) $\times 110,000$.

FIGURE 3 A tubules negatively stained with uranyl acetate. The A tubules were prepared by thermal fractionation of doublet tubules. $\times 135,000$.

FIGURE 4 Helical particles from an axonemal preparation that had been briefly digested with trypsin followed by addition of glutaraldehyde (negatively stained with uranyl acetate). *a*, $\times 33,000$; *b*, $\times 30,000$.

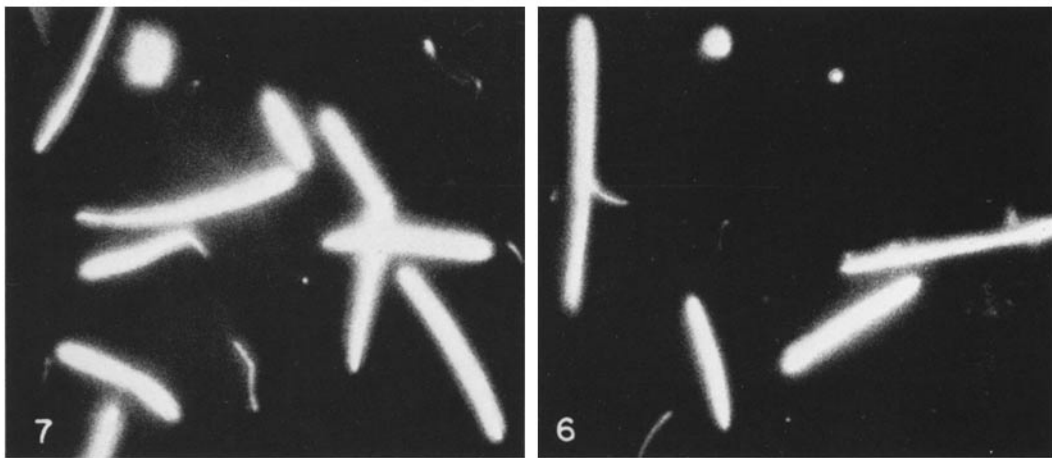
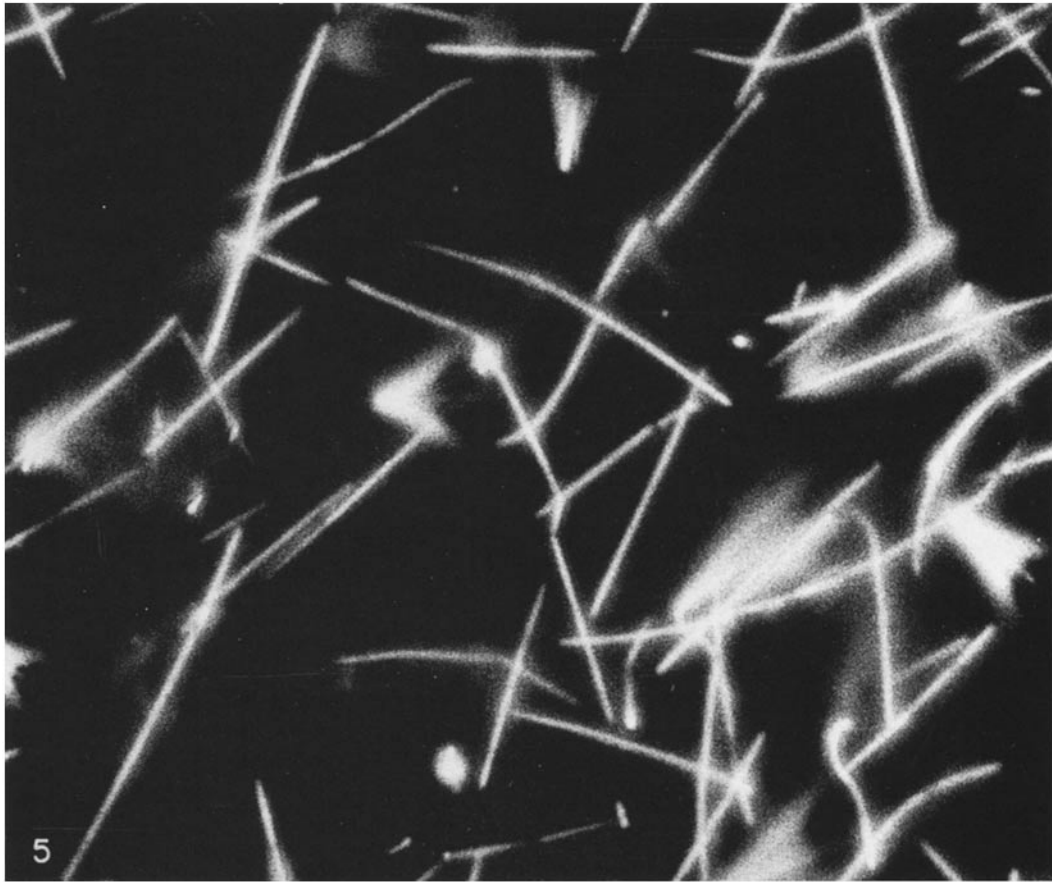


FIGURE 5 Axonemes in suspending media. $\times 1,900$.

FIGURE 6 OF's in suspending media. $\times 1,600$.

FIGURE 7 OF's in 2% glutaraldehyde, 10 mM PO_4 , pH 7. $\times 1,500$.

FIGURE 8 Axonemes in 2% glutaraldehyde, 10 mM PO_4 , pH 7. $\times 1,700$.

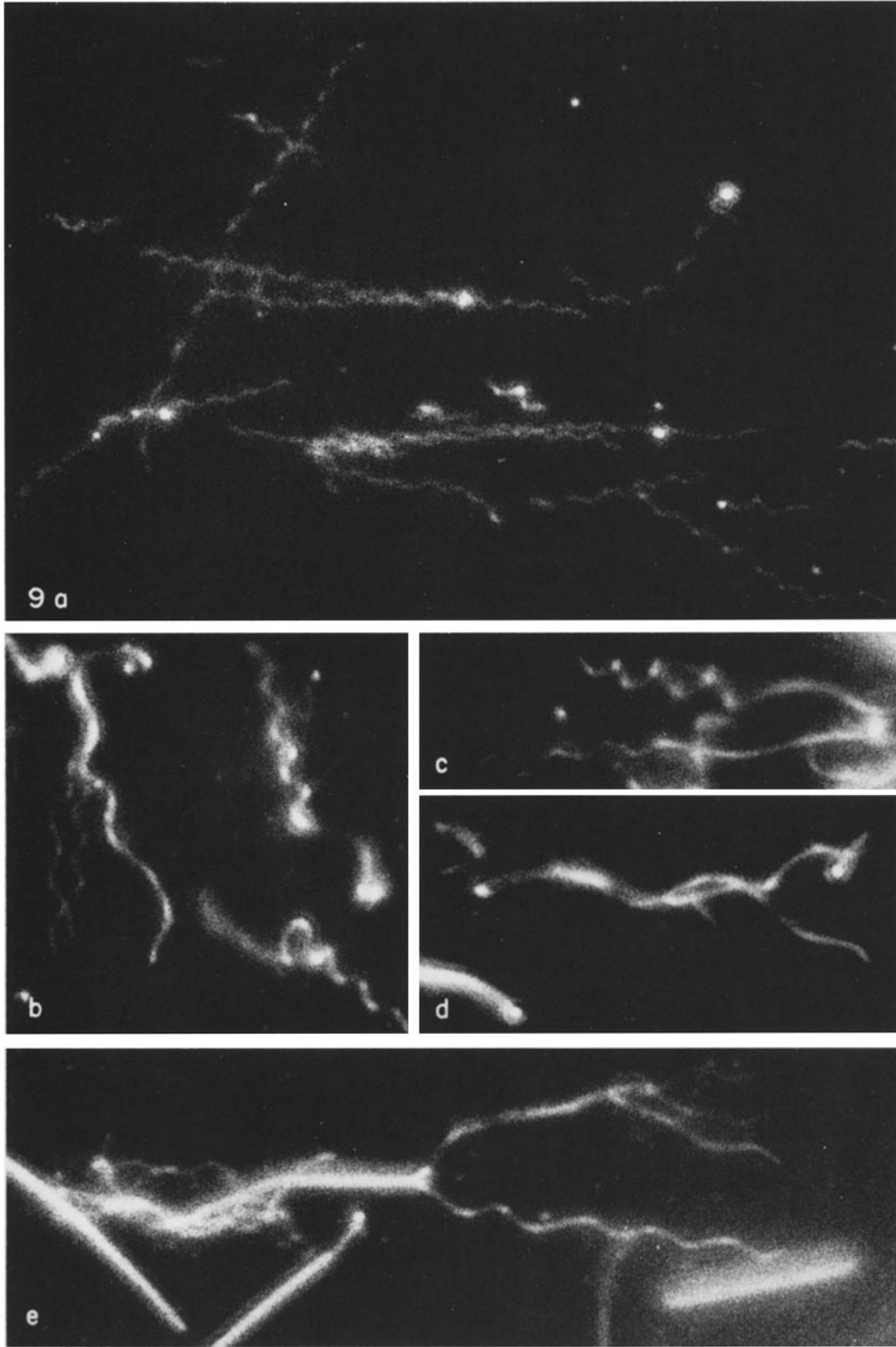


FIGURE 9 Axonemal digests in 2% glutaraldehyde, 10 mM PO_4 , pH 7. *a*, $\times 2,200$; *b-e*, $\times 2,800$.

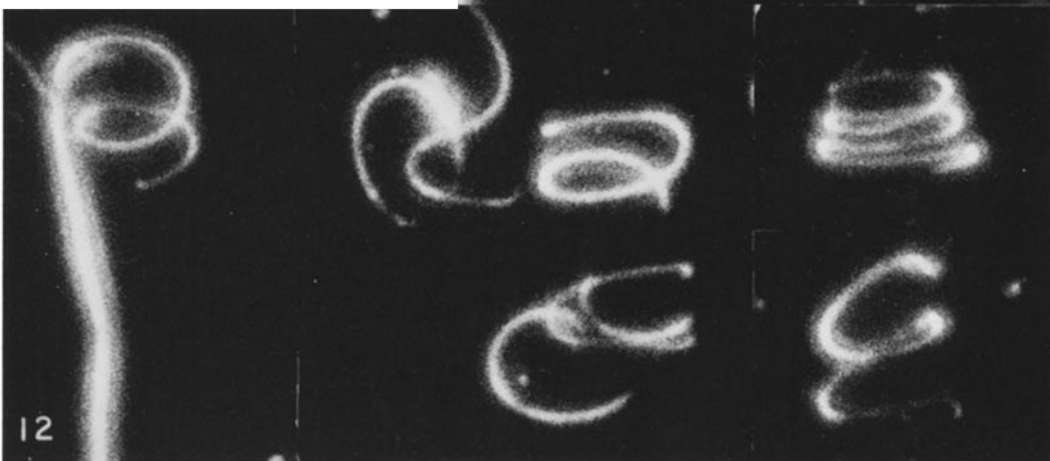
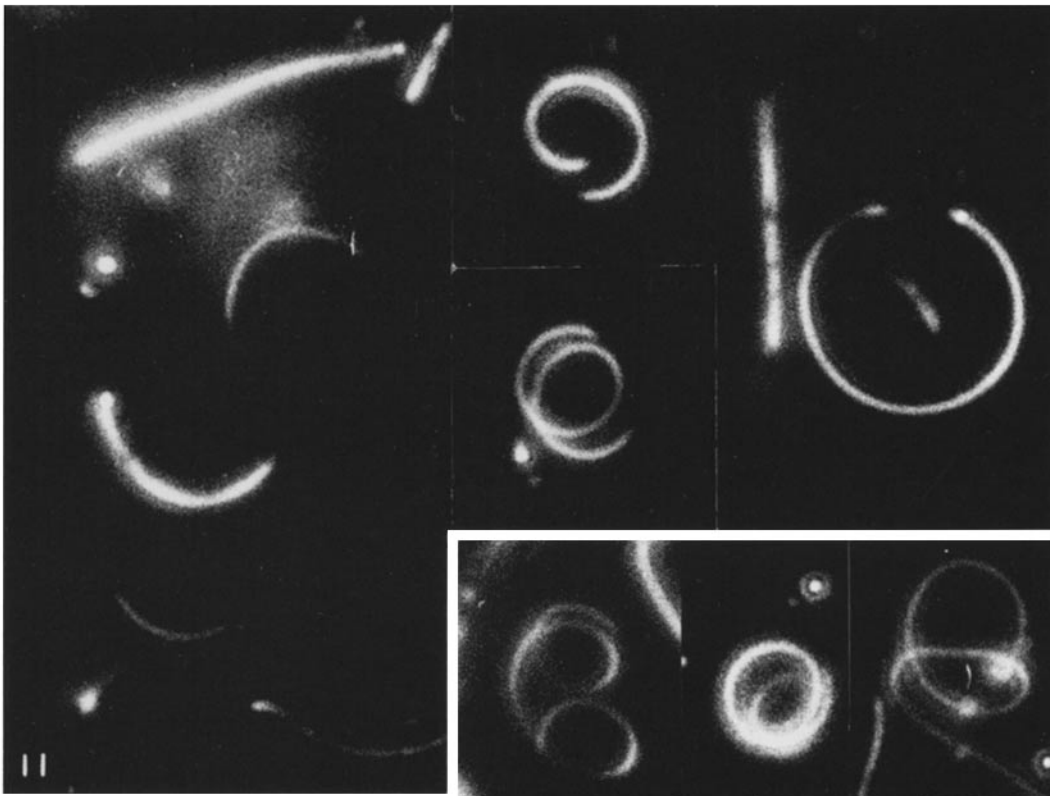
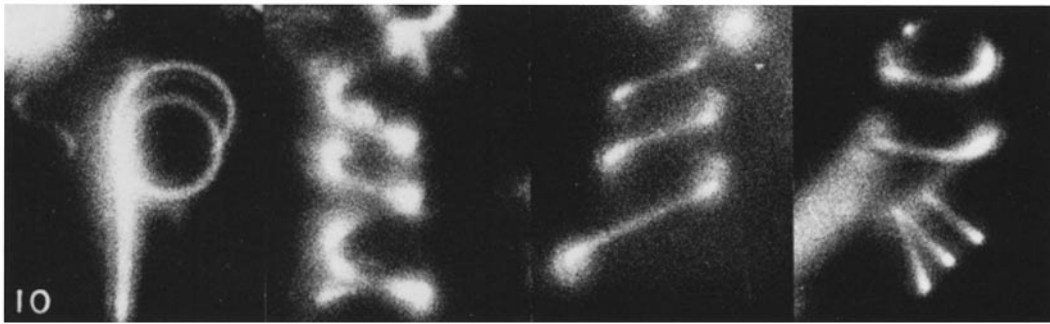


FIGURE 10 Axonemal digests in 6 mM CaCl_2 , 1×10^{-4} M GTP. $\times 2,800$.

FIGURE 11 OF's in suspending medium after dialysis against 2 mM Tris, 10^{-4} M EDTA, 10^{-4} M DTT. $\times 2,800$.

FIGURE 12 Axonemal digests in 1×10^{-3} M colchicine, 1×10^{-4} M GTP. $\times 2,800$.

TABLE I
Solutions in which Digestion Products Appeared as Helices or Coils

Reagents	Extent of digestion			Comments
	90%	70%	50%	
2% Glutaraldehyde	+, MA	+++ , SA	+++ ,	Usually quite regular
9 mM MgCl ₂	0, MA	+++ , SA	+, DP	Not very regular
6 mM CaCl ₂	+, MA	++ , SA	+, DP	More regular than in MgCl ₂
1 × 10 ⁻³ M Colchicine + 10 ⁻⁴ M GTP	+, SA	++ ,	++ , DP	Irregular, tend toward coils
1 × 10 ⁻³ M Vinblastine	+, MA	+++ ,	+, DP	Causes dissociation
0.05% Sarkosyl	++ , SA	+++ ,	DP	
4% Formaldehyde	+, MA	+,	+	Causes some aggregation
10% Formaldehyde	0, MA	++ ,	++	
0.2% PTA	++ , SA	+,	DP	Causes dissociation
Tris-HCl	+, MA	++ ,	—	Misshapen, but not helices
SM	0, MA	0, SA	—	

Plus signs indicate fraction of helical coils in preparation (e.g., +, few; +++, many or most).

MA and SA indicate portion of axonemes or fragments in preparation (or where appropriate OF's or their fragments, MA, many; SA, few).

DP, disintegration products, usually very low intensity scattered radiation, short, irregular shapes.

The results presented in this table are a summary of the observations made in solutions containing these reagents. In addition the solutions contained buffers, either phosphate or Tris-HCl and in some cases also KCl. In those instances where observations were made of the effect of a reagent in more than one buffer these results represent an average or optimal interpretation. Dark-field micrographs of the various conformations observed are shown in Figs. 7-15.

EFFECT OF REMOVAL OF DYNEIN: Removal of dynein by extraction noticeably increased the tendency of digestion products to undergo conformational alterations to helices or coils.

EFFECT OF COMPOSITION: EDTA, DTT, and Mg⁺⁺ ions all seemed to repress helix formation somewhat although in no case was there complete absence of any conformational alteration. (Note that the solution used for storing the axonemes and occasionally digests contained all three of these reagents.)

From the relative intensity of the particles observed by dark-field microscopy it appeared that many of those assuming helical or coiled conformations were doublet tubules or possibly pairs of doublet tubules in addition to larger groupings. In general the most intense particles (axonemes or fragments produced by transverse dissection) were relatively resistant to the distortion required for helix formation.

Electron Microscope Observations

Electron micrographs of negatively stained specimens of typical helical structures are shown in Figs. 4 *a* and 4 *b*. In both of these figures the filaments apparently consist of two doublet tubules linked together, and although an indication of protofilament substructure is apparent no further structural details can be perceived. Lateral projections are quite evident on the structure in Fig. 4 *b*. These projections are no doubt the same as those observed on critical point dried and shadowed specimens shown in Fig. 16.

The appearance of negatively stained doublet and singlet tubules is shown in Figs. 2 and 3, respectively. Note that the protofilament structure of the A tubules is apparent but that it is not in the doublets. The apparent diameters of the doublet and singlet structures are approximately 30 nm and 22 nm, respectively.

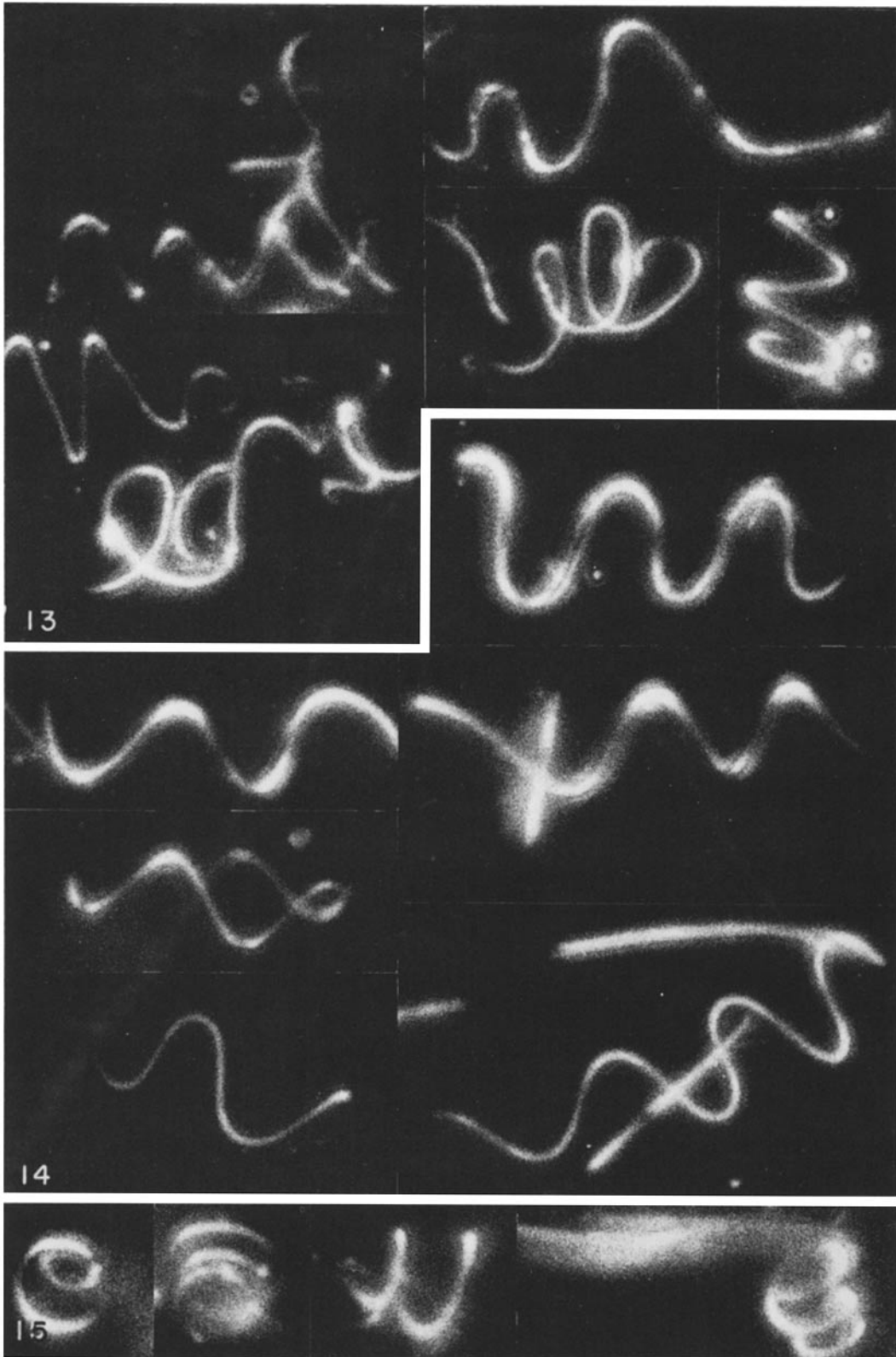


FIGURE 13 Axonemal digests in 9 mM $MgCl_2$, 1×10^{-4} M GTP. $\times 2,800$.

FIGURE 14 Axonemal digests in 0.05% Sarkosyl. $\times 2,800$.

FIGURE 15 Axonemal digests in 25 mM Tris-100 mM KCl, pH 8. $\times 2,800$.

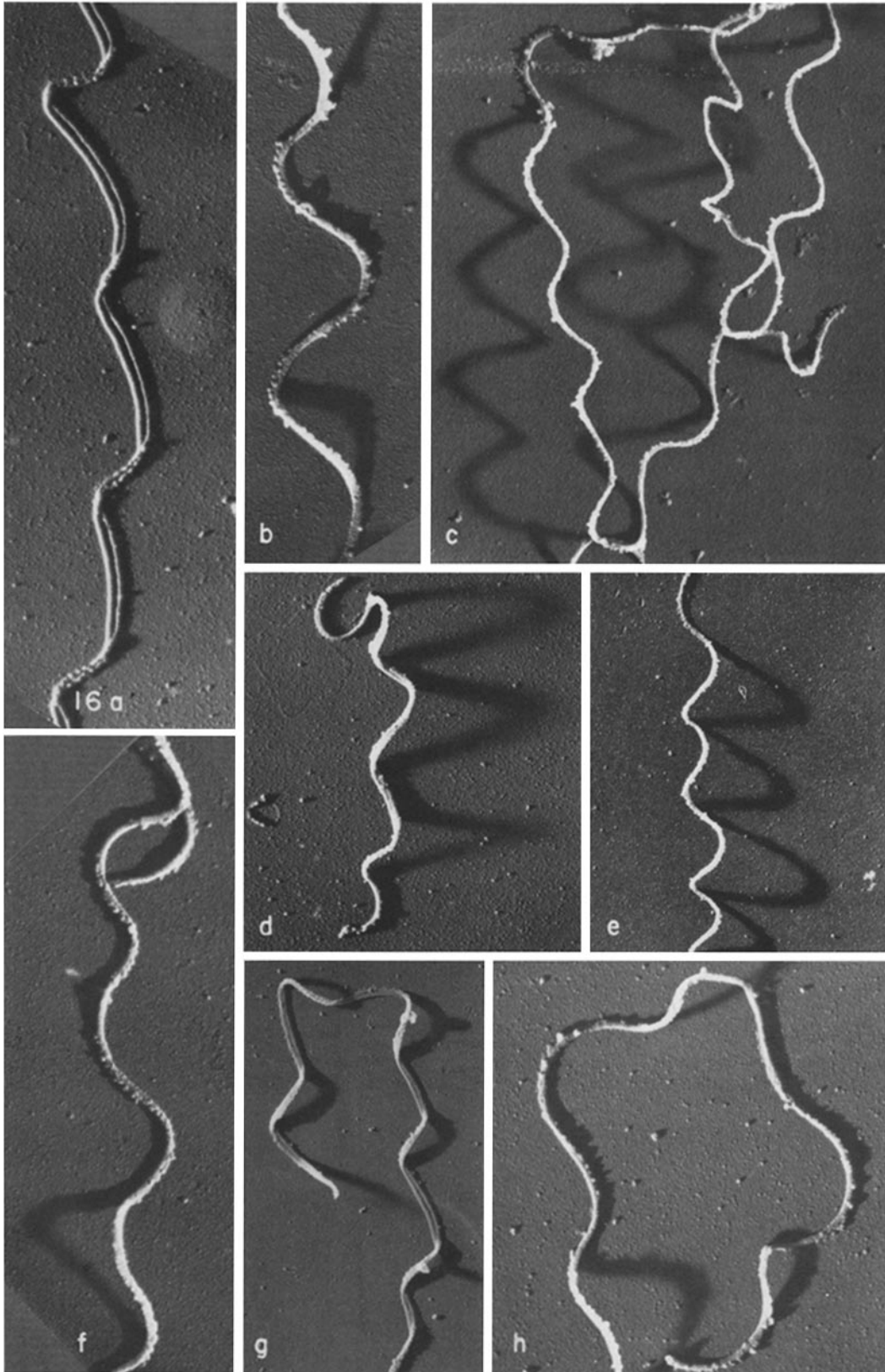


FIGURE 16 Electron micrographs of particles from axonemal digests in glutaraldehyde. Specimens prepared by critical point procedure followed by shadowing with platinum. Magnifications as follows: *a*, $\times 12,000$; *b*, $\times 25,000$; *c*, $\times 17,000$; *d*, $\times 13,000$; *e*, $\times 14,000$; *f*, $\times 25,000$; *g*, $\times 13,000$; *h*, $\times 21,000$.

Dramatic proof of the helical nature of the filament structures is provided in observations on specimens prepared by critical point drying followed by shadowing (see especially Figs. 16 *c, d, e*). In many instances the helical structures seem to be individual doublet tubules; (Figs. 16 *b, c, e, f, h*) whereas, others appear to consist of a pair of doublet tubules (Figs. 16 *a, d, g*). Measurement of the filament diameter on a number of structures yielded values of ca. 64 nm and ca. 110 nm, respectively. The average pitch of the helices is ca. 1.1 μm and the helix diameter is approximately 0.5 μm . Both right- and left-handed helices have been observed.

Also to be noted are the globular projections along the length of the structures. Quite clearly these projections occur only on one side of the filaments and generally this is the side with the larger radius of curvature. Accurate determination of their dimensions and spacing was not possible, but a number of measurements yielded the rough value of 56 nm for their average separation.

Effect of Digestion on Activity

To obtain further information about the effect of digestion on the axonemes the variation in ATPase activity of axonemal suspensions digested for different lengths of time was followed in a systematic manner. Preparations of axonemes were digested and aliquots were withdrawn at various intervals of time to be added to inhibitor. The ATPase activities of these aliquots were then determined on the pH-stat. The results for one such experiment are presented in Table II. It is evident that there is a general increase in activity as the digestion proceeds, although it is not strictly monotonic (probably attributable to experimental error). Comparison of the last two columns shows that there was relatively little change in turbidity after inhibition of the digestion.

Additional information on the nature of the increase in activity was obtained from a series of experiments in which aliquots were withdrawn from digests, added to inhibitor, and centrifuged: after decantation of the supernatant the pellet was resuspended in SM and the activities, concentrations, and volumes of all the fractions were determined. The distribution of protein, specific and total activities in each fraction were determined and compared with the original axonemal preparation as well as the digest mixtures. In addition, aliquots of the supernatant and pellet fractions were run

TABLE II
Variation of ATPase Activity with Extent of Digestion

Aliquot no.	Time of digestion	Specific activity*	Relative activity†	% OD _i ‡	
				I	F
	<i>min</i>				
0	0	0.24			
0 + 1	0	0.26			
1	1	0.32	1.22	95	91
2	3	0.31	1.19	89	85
3	5	0.30	1.16	82	80
4	7	0.40	1.52	81	75
5	9	0.37	1.4	77	73
6	11	0.44	1.68	74	70
7	15	0.44	1.68	70	64
8	19	0.50	1.9	66	58
9	30	0.51	1.95	58	54
10	40	0.49	1.84	54	49

15 ml of axonemes (0.34 mg/ml) digested with 2×10^{-3} mg trypsin. 1 ml aliquots withdrawn and added to 0.2 ml of inhibitor (10^{-1} mg/ml) at 0°C. 500- μl aliquots assayed for activity on pH-stat. * Activity in micromoles P_i per milligram per minute.

† Determined from rate of base consumption.

‡ I determined from initial optical density and optical density at time aliquot was removed for addition to inhibitor. \therefore OD_i = zero value. F determined from optical densities of aliquots in inhibitor at completion of experiment. \therefore OD_i = value of initial axonemal aliquot that had been added to inhibitor. This will be in error by the dilution factor due to trypsin, but that is insignificant.

in sodium dodecyl sulfate (SDS) gels to characterize their constituents. Finally, aliquots from the pellet fractions were fixed, embedded, and sectioned for observation in the electron microscope. These results are discussed in a later section.

The results from four digestion experiments are summarized in Table III. From these results the following general observations can be made:

(a) The specific activity of the digests increases with increasing extent of digestion.

(b) The activity (both specific and total) of the supernatant fraction increased with extent of digestion, whereas that of the pellet decreased.

(c) The quantity of protein in the supernatant fraction increased and correspondingly the quantity in the pellet fraction decreased with increased digestion.

TABLE III
Summary of Results from Digestion Experiments on Axonemes for Varying Lengths of Time with Trypsin

		Average percent recovery of:		Average relative specific activity
		Total activity	Total protein	
A.				
Digest	One	84	88	0.93
	Two	112	91	1.0
	Three	116	92	1.39
B.	Total activity, total protein			
Supernatant	One	24	23	0.96
	Two	66	34	1.96
	Three	88	42	3.19
Pellet	One	68	70	0.87
	Two	49	62	0.73
	Three	20	53	0.55
Percent recovery =				
Total activity ([supernatant + pellet]/[digest])				
Total protein ([supernatant + pellet]/[digest])				
Percent total quantity =				
Total activity ([supernatant or pellet]/[supernatant + pellet])				
Total protein ([supernatant or pellet]/[supernatant + pellet])				
Relative specific activity =				
Specific activity ([supernatant, or pellet or digest]/[axonemes])				

This table presents the averaged results from four independent experiments.

(d) There was an approximate balance of the total activities and quantities of protein in the various experiments.

The specific activity of the pellet fell to about 0.1–0.2 $\mu\text{M P}_i/\text{mg}$ per min, which is very slightly greater than for axonemes extracted with KCl (average of 0.10 $\mu\text{M P}_i/\text{mg}$ per min for five experiments). Thus, removal of activity from axonemes by digestion is approximately as effective as by treatment with KCl. On the other hand, dialysis of axonemes against low ionic strength buffer reduces their specific activity to practically zero. Stated somewhat differently, KCl extraction removes 81% of the total axonemal activity (as does extended digestion) while low ionic strength

extraction removes 98% of it; however, the quantities of protein removed by KCl and low ionic strength are respectively 39% and 41%, while in the digestion process approximately 45% of the axonemal protein goes into the soluble phase.

Although the specific activity of both the supernatant and the digest mixture always increased with increasing digestion, for some limited digests (e.g., digest one, Table III) the specific activity of the digest was less than that of the undigested axonemes. There was some indication that this depended on the level of specific activity of the axonemes but the correlation was not precise.

Gel Electrophoresis

Aliquots from both the supernatant and pellet fractions of the digest and extraction experiments were run in SDS-acrylamide gels.

Results from one experiment are shown in Figs. 17 *a* and *b*.

In Fig. 17 *a* the band patterns resulting from gel electrophoresis of supernatants (gels 3, 4, 5, 6) from the digest are shown, while the patterns from the pellets (gels 1, 2, 3, 4) from the same digests are shown in Fig. 17 *b* (in Fig. 17 *a* gels 1 and 2 and in Fig. 17 *b* gels 5 and 6 are controls; dynein and axonemes, respectively). The relative sp act for the supernatants were 1.28, 1.10, 2.72, and 4.48, while for the pellets they were 1.10, 0.93, 0.72, and 0.59 (gels 3, 5, and 6 in Fig. 17 *a* and 1, 3, and 4 in Fig. 17 *b* correspond to supernatants 1, 2, and 3 and pellets 1, 2, and 3, respectively in Table III).

From the various controls it can be seen that under the conditions used here the band nearest the top of the gels (i.e., slowest moving) is characteristic of dynein. The fairly intense band in the middle of the gels is presumably due to tubulin, although in these experiments positive identification was not attempted. Visual examination of the gel electrophoresis patterns provides a basis for two generalizations:

(a) With increasing digestion of the axonemes the slow-moving band (characteristic of dynein) increases in intensity on the gels of the supernatants. Correspondingly, these samples have increasingly larger relative specific activities.

(b) In the gels of the insoluble fractions, this band decreases in intensity as does the relative specific activity of the sample.

The net conclusion from these observations is that the activity released into the supernatant phase is the result of cleavage of all or part of in-

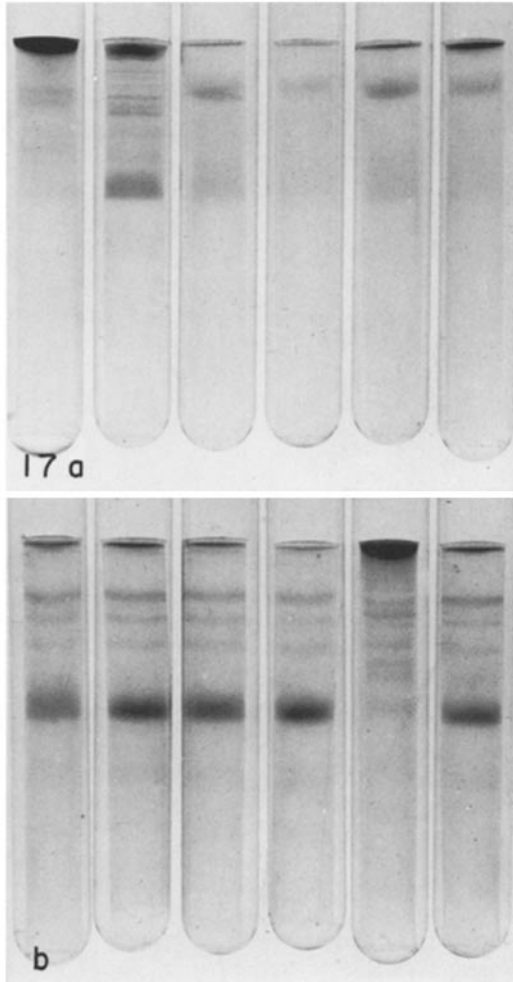


FIGURE 17 SDS-gels of supernatants and pellets from tryptic digests of axonemes. Gels were 8% acrylamide, reservoir buffer was ca. pH 7.2, phosphate. 200 μ l of sample applied to each gel. (a) Samples on gels are respectively: dynein, axonemes, supernatants 1, 1 a, 2, and 3. (b) Samples on gels are respectively: pellets from digests 1, 1 a, 2, 3; dynein, axonemes. Digests 1, 2, 3 are comparable to those listed in Table III.

dividual dynein arms from the doublet tubules. Further, the specific activity of this released protein evidently increases with increased digestion. Gels 6 (Fig. 17 a) and 4 (Fig. 17 b), which show this effect quite clearly, correspond to the release of about 45% of the axonemal mass into the supernatant phase and correspond to the conditions of digest three for the experimental results presented in Table III.

Electron microscope observations on sectioned

specimens prepared from the pellet fractions showed that in digest number one the axonemes appeared to be largely intact, except that one tubule of the central pair was frequently missing. Occasionally missing arms were noted on the doublet tubules and the radial spokes were frequently disrupted. The observations were not sufficiently penetrating to allow comment concerning any effect on the nexin links.

In specimens prepared from digest two it was noted that in addition to the apparent complete removal of the central pair, radial spokes were usually absent, occasionally there were missing arms and tubules, and many of the axonemes were broken down to groups of doublet tubules and even individual doublet or singlet tubules (with and without arms).

Specimens prepared from pellets from digest three were observed to contain only isolated doublet or more generally singlet tubules. Arms were evident on many of the tubules.

No effort was made to obtain a precise correlation between appearance of the insoluble fraction and extent of digestion since this has been studied in detail by Summers and Gibbons (1973).

DISCUSSION

Variation of Turbidity with Digestion

The decrease in turbidity with increasing digestion can be ascribed to two factors: (a) the decrease in mass of any particular particle due to the release of small fragments into the soluble phase of the digest solution and (b) the decrease in size (both mass and volume) of a particle due to its separation into two parts whose combined masses and volumes are more or less equivalent to that of the original particle. Unfortunately, the paucity of data as well as theoretical understanding of the scattering process for such suspensions prevents completely satisfactory interpretation of the observations (but see Gibbons, 1965 b).

The increase in both rate of decrease and extent of decrease in turbidity for proteolysis occurring in the presence of ATP is due to induced dismemberment of the particles by active forces (see Summers and Gibbons, 1971). In the current investigation it was noted that in digests to which ATP had been added many fewer large fragments were observed in dark-field preparations as compared with the same digest without ATP.

Effect of Solution Composition on Particle Conformation

Although groups of tubules in helical conformations were first observed in glutaraldehyde, later observations in other solvents showed that the formation of helices was a general phenomenon. Perhaps, the most striking conclusion to arise from these studies is the wide variety of solution compositions under which the helical conformation occurs, suggesting a tendency for the axonemal components to spontaneously undergo a conformational transformation when not constrained in the axonemal unit. Specific conditions for suppressing helix formation were not actually found, although the solvent used as suspending and storage medium for most of the experiments fortuitously turned out to be the most effective in repressing their formation. The repressive effect seemed to be due to the combined presence of Mg^{++} ions, EDTA, and DTT, each of which was partially effective in suppressing helix formation. The only other conditions under which no helices were found were those that led to essentially complete disruption of the axonemal structure (urea, G-HCl, high pH, etc.) or conditions that led to extensive aggregation (most notably low pH).

It is important to note that in spite of the general tendency toward helix formation under a wide range of solution conditions, when undigested OF's or axonemes were exposed to glutaraldehyde only a minimal fraction of helices (see Figs. 7 and 8) were observed. Large numbers of helices were formed only when *digests* were exposed to glutaraldehyde or other solutions as listed in Table I. Thus, 0.5 M KCl, PTA, Sarkosyl, colchicine, urea, G-HCl, ethanol, low ionic strength neutral salt solutions, and high pH all enhanced dissociation of partially digested axonemes. That the mechanism of dissociation was the same in all cases seems most unlikely. The effects on particle conformation of pH, dissociating agents, and ATP were all consistent with the suggestion that once the axonemal structure is disrupted by proteolysis, the reagents enhanced dissociation of the basic unit allowing the components to undergo the conformational transition. The helical conformation is a consequence of both solution composition and the effects of digestion. An increase in digestion led to a larger fraction of the particles having a helical conformation.

Low ionic strength appeared to have two effects

on axonemes or OF's. Exposure of partially digested OF's or axonemes to low ionic strength resulted in the appearance of helical coils in proportions that correlated roughly with the extent of digestion, suggesting that with reduced charge shielding, partially digested particles dissociate and the dissociation products undergo conformational alterations. For nondigested axonemes or OF's a tendency toward curvature or formation of coils was noted at low ionic strengths (Fig. 11). Helix or helical coil formation was probably prevented by the circumferential nexin fibers and the radial spokes.

Other workers have also noted some sensitivity of doublet tubules to solution conditions. Thus Henley et al., 1969, pointed out that when exposed to PTA, the flagella of spermatozoa from *Mesostoma* became transformed into helical conformations. Helical conformations of tubular elements were also observed in spermatozoa of *Chaetopterus* (Costello and Henley, 1969). However, the general nature of axonemes seems to be repressive to extensive twisting or bending as encountered in these particular examples.

Nature of the Conformational Alteration

The integrity of intact axonemes is maintained largely by the circumferential nexin fibers and the radial spokes. Two of the observations made here have also suggested that interaction between the dynein arms of the A tubule and the B tubule may play a role in stabilizing the axonemal structure. The first of these was the observation that partially digested axonemes whose dynein had been removed by KCl extraction readily disintegrated and formed helices under conditions in which unextracted controls did not. Secondly, the greater flexibility and tendency for OF's to undergo structural transformations supports the same conclusion. Efforts to show a direct interaction by determining the effect of B tubulin on the ATPase activity of dynein or axonemes were not successful. In view of the probable importance of precise structural arrangements in any such interaction the negative result is not surprising. The helical or coiled particles observed by dark-field microscopy are usually less intense than the axonemes or axonemal fragments. In preparations of highly disintegrated axonemes where practically all the particles observed were helical, their intensity was frequently quite low, suggesting they might be doublet tubules or possibly pairs of doublet tubules. The electron micro-

scope evidence shows that this is the case. Thus, it seems that the ability to assume the helical conformation resides in the individual doublet tubules and is not the result of interaction between them. The fact that OFs or tubules derived from OF's form helices also supports this interpretation.

Presumably the formation of the helical conformation by doublet-tubules would require rearrangement of the subunit parts. Thus, the longitudinally running protofilaments would have to undergo relative displacement to accommodate the transformation. Assuming the doublet tubules behave as suggested by Lowy and Spencer (1968), a 16% shift has been estimated between the longest and shortest protofilaments (the doublet tubule was approximated as a cylinder) in the helices observed. At present the protofilaments are believed to be made up of globular units of the protein tubulin, whose precise arrangement in the tubule can only be conjectured. In addition to the usual longitudinally running protofilaments, the appearance of a helical pattern has been noted by Burton (1966 *a, b*, 1968, 1970) and Thomas (1970) who pointed out that transformations between these two structural arrangements could be induced by varying the tubule's environment. No suggestion was made that a larger conformational alteration of the tubules might occur as a result of the substructural transformations, although such a conformational alteration has been observed in specimens treated with glutaraldehyde and PTA (Costello and Henley, 1969; Henley et al., 1969). Efforts to detect substructural arrangements in the helical structures observed here were not successful. There also remains the possibility that the two patterns observed (helical and longitudinal) are a result of different ways of staining the same arrangement of subunits.

Why the tubules should assume a helical conformation once the cross-linking constraints are removed is not clear. However, as shown by Lowy and Spencer, 1968, only small rearrangements in subunit packing would be required to accommodate the conformational alterations observed. Such subunit rearrangements might be a consequence of several interrelated factors: construction of the doublet tubules from nonequivalent subunits (Asakura and Iino, 1972; Everhart, 1971; Feit et al., 1971; Lowy and Spencer, 1968; Olmstead et al., 1971), structural alterations to the tubular system by the enzyme, or the existence of axial stresses in the tubular structure. Rikmenspoel (1971) has

suggested that a thin rod subject to an axial load will assume a conformation similar to the wave forms found in some sperm. In view of not only the geometrically nonsymmetrical cross section of doublet tubules but their nonsymmetrical mass distribution as well, axial tension might result in the helical conformations observed. Increased stiffness resulting from the projections could account for their appearance on the side with the largest radius of curvature (and would be consistent with Lowy and Spencer's hypothesis, 1968).

As mentioned previously, the parameters of the helices vary noticeably but in general the pitch is from 1 to 8 μm and the helix diameter is approximately half the pitch. The measured filament diameters are consistent with their being individual doublet tubules. In this regard it should be pointed out that the measurements on negatively stained preparations are more closely in accord with previous determinations of singlet and doublet tubule dimensions.

Of considerable interest is the observation of both right- and left-handed helices. Unfortunately it cannot be ascertained whether the handedness is a fortuitous consequence of the preparative procedure or whether some doublet tubules form only right-handed helices while others form only left-handed helices. Such tendencies may relate to the pattern of beat of the intact sperm tail (Gray, 1962; Rikmenspoel, 1962).

The dimensions and separation of the projections, in addition to their apparent location on the filaments consisting of two doublet tubules, suggest that they are probably the remnants of radial spokes rather than dynein arms. Warner (1970) has presented evidence that radial links occur as pairs (340 \AA apart) spaced at 640 \AA intervals along the central sheath (*Chlamydomonas reinhardtii*). This distribution agrees reasonably well with the approximately 500 \AA –600 \AA separation observed here with the additional assumption that the pairs have collapsed together in preparation and are observed as single entities. Note might also be made of Grim's (1970) observations of projections (nodes) on doublet tubules from a variety of sources. Qualitatively the nodes are similar in appearance to the projections observed here and their spacing (44–100 nm) agrees more closely with that of the radial link pairs (64 nm) than the dynein arms (\sim 10–20 nm). Also the fact that in pairs of doublet tubules (this study and Grim) or larger groupings (Grim) the nodes

project from the same side of the tubules rather than from one doublet towards another, argues against their being the dynein arms. However, better preparations and more precise measurements will be required to definitively establish the identity of these structures.

Effect of Digestion on Axonemes

The results of the digestion experiments (Table III) and the gel electrophoresis experiments provide consistent evidence that dynein is released during tryptic digestion of axonemes. When approximately 25% of the axonemal protein appears in the soluble phase, this fraction has a specific activity of $0.3 \mu\text{M P}_i/\text{mg per min}$, which is about one-third that of dynein prepared by extraction (when compared under the same experimental conditions). Assuming the activity of the released dynein is approximately the same as dynein prepared by extraction, this suggests that ca. 15% of the active axonemal dynein has been released from axonemes when digestion has proceeded to this extent. This estimate may be too high in view of the apparent increase in specific activity of the dynein released from axonemes by proteolysis.

The release of dynein during tryptic digestion of axonemes was observed by Mohri et al. (1969), who estimated that when 33.5% of the protein appeared in the supernatant phase it contained 47.9% of the ATPase activity. Although direct comparison of the results obtained here with those of Mohri et al. is not possible, the same general trend is shown in each. There is an indication that the relative specific activity of the supernatant phase found in our experiments is probably somewhat greater than found by Mohri et al. This may reflect the increased specific activity of dynein as a consequence of digestion as observed here, but which was not observed or at least commented upon by Mohri et al. Due to differences in procedure and technique more precise comparison of the results is probably not warranted.

Comparison of the results of the digestion experiments with those of the extraction experiments reveals that for release of about 40% of the axonemal protein into the supernatants the specific activities of these fractions were 1, 0.7, and $1.2 \mu\text{M P}_i/\text{mg per min}$ for the digest, KCl, and low ionic strength experiments, respectively. Gel electrophoresis results suggested that the supernatant from low ionic strength extraction probably contained a smaller fraction of contaminants than

either of the other two supernatants, although the distinction between the two extraction experiments is tenuous at best. Within the limitations of comparing the results of these experiments with those previously reported (Gibbons, 1965 *a*) it is noted that electron microscope observations on the digest pellet revealed extensive disintegration of axonemes, whereas extracted axonemes appeared largely intact. All these results suggest that the mechanism of dynein release from axonemes is rather different for digestion and extraction and that extraction is probably preferable if preparation of dynein is the objective.

The morphological effects of the initial phases of digestion have been studied in detail by Summers and Gibbons (1973) and digestion of a "9 + 1" axoneme has been studied by Thomas (1971). In this investigation the principal objective has been clarification of the mechanism whereby proteolysis with trypsin makes possible conformational alterations of the axoneme or probably more precisely, its digestion products. Although the initial locus of attack has not been sought, it is clear that by the time digestion has released 25% of the axonemal mass into the supernatant fraction dynein can also be detected in that fraction as evidenced by its ATPase activity and its appearance as a band on polyacrylamide gels. At this stage of digestion the principal morphological observation is that all or part of one of the central pair of tubules has been removed and that the radial spokes are usually disrupted but not absent. Whether proteolysis results in removal of the entire arm from the tubular structure or only a portion of it containing activity has not been ascertained. The gel electrophoresis results would support the former possibility while the indications of an increased specific activity of the supernatant protein would be consistent with the latter—quite possibly both occur. Further proteolysis results in: release of additional protein into the supernatant with an increased specific activity, a diminution of the fraction of protein in the pellet with a concomitant decrease in its specific activity, increase in the quantity of dynein observed in gels of the supernatants, decrease in the quantity of dynein observed in gels of the pellet fractions, and extensive disruption of the axonemal structure as revealed by electron microscope observations on specimens of thin sections prepared from the pellet fractions. That axonemal components display different susceptibilities to proteolysis has been shown previously in a number of investigations

using pepsin (Behnke and Forer, 1967; Burton, 1968; Silveira, 1968) and has formed the basis for a proposal of different classes of microtubular elements.

CONCLUSION

The series of experiments performed here strongly suggest that the axonemal structure exists under tension so that when the physical restraints of the circumferential nexin fibers and radial cross-links are removed the doublet tubules undergo spontaneous conformational alterations. That the removal of these constraints is probably the initial action of proteolysis has been shown by Summers and Gibbons (1973). Here it has been found that although by the time 25% of the axonemal mass has been released into the soluble phase there is only limited apparent morphological disruption of their structure, ATPase activity is detectable in the supernatant fraction. It has been estimated that less than 8% of the axonemal mass (which may represent approximately 15% of the total axonemal dynein) is released at this stage. Although some evidence has been obtained in support of the contention that the dynein arms aid in maintaining the integrity of the axonemal structure, the extent of tryptic digestion correlates most strongly with the fraction of particles undergoing the conformational transformation to coils and helices. Thus, the major factor in proteolysis is disruption of the nexin and radial constraints rather than removal of dynein. These observations raise the intriguing question of how the axonemal structure is assembled under strain and the role this may have on the mechanism of action of the motile apparatus.

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