

PROPERTIES OF THE PROTEIN SUBUNIT OF CENTRAL-PAIR AND OUTER-DOUBLET MICROTUBULES OF SEA URCHIN FLAGELLA

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ABSTRACT

The subunit protein has been isolated from the central-pair and outer-doublet microtubules of sea urchin sperm tails. Both proteins have a sedimentation constant of 6S and a molecular weight of 120,000. Both are converted to a 60,000 molecular weight species by denaturation in 6 M guanidine hydrochloride and reduction with mercaptoethanol. The reduced-alkylated proteins have the same R_f on disc electrophoresis, and the same amino acid composition, which is very similar to that of muscle actin. The central-pair protein has one binding site for colchicine per 120,000 g. Both proteins appear to have a guanine nucleotide binding site, but the ability to bind GTP in solution has been demonstrated only for the central-pair protein. Although 1 mole of guanine nucleotide is bound per 60,000 g to outer-doublet tubules, the protein obtained by dissolving the doublets at pH 10.5 has lost the guanine nucleotide-binding site and also shows little or no colchicine-binding activity. Comparison of the properties of the isolated protein with electron microscopic evidence on structure of microtubules suggests that the chemical subunit ($M = 120,000$) consists of two of the 40 A morphological subunits.

INTRODUCTION

The central pair and outer-nine doublet tubules of cilia and flagella are regarded as members of the general class of structures referred to as microtubules. Previous work showed the central pair to consist of a protein subunit, of sedimentation constant 6S, with a specific binding site for colchicine (1). A colchicine-binding protein with similar properties was also found in mitotic apparatus, in the cytoplasm of dividing cells, and in nervous tissue (2, 3).

The present work describes the isolation and characterization of the protein subunits of central-pair and outer-nine tubules of sea urchin sperm tails. In their physical properties and amino acid compositions, both proteins are very similar and correspond closely to the protein obtained from

mammalian brain.¹ A characteristic feature of tubule protein obtained from four different phyla is the binding of guanine nucleotides. The protein also appears to show some similarities with the muscle protein actin, which has a similar amino acid composition and binds adenine nucleotides.

The properties of the tubule proteins reported here are very similar to those recently described by Renaud et al. (4) for *Tetrahymena* cilia.

MATERIALS AND METHODS

Preparation of Proteins

Strongylocentrotus purpuratus sperm (Pacific Biomarine Supply Co., Venice, Calif.) were generally used for

¹ R. Weisenberg, G. G. Borisy, and E. W. Taylor. In preparation.

isolation of tubule protein. The procedure for isolation of sperm tails and the preparation of central-pair protein has been described previously (1).

Outer-doublet protein was obtained by the extraction of freshly amputated material with 0.6 M KCl at pH 7 for 5 hr for the removal of the dynein and central-pair proteins. Examination of sectioned pellets in the electron microscope showed only outer-doublet tubules remaining. The doublets were then dialyzed for 24 hr against 10^{-2} M mercaptoethanol in 0.05 M carbonate buffer at pH 10.5. About 90% of the remaining protein was extracted in this step. We centrifuged the preparation for 4 hr at 60,000 rpm to free it of high S materials.

Protein preparations were denatured by dialysis for 24 hr against a solution of 6 M guanidine hydrochloride, 0.4 M KCl, 0.2 M mercaptoethanol, 0.01 M tris buffer, pH 8.0. The guanidine hydrochloride (Eastman Chemical Co., Rochester, N.Y.) was purified by crystallization from hot methanol.

The protein was alkylated by reaction with 0.3 M iodoacetic acid for 1 hr, while temperature and pH were maintained at 20°C and pH 8, respectively. This step was carried out under a nitrogen atmosphere in a light-shielded flask.

Protein content was determined by the Lowry method (5) which was calibrated refractometrically by determination of the concentration of a purified sample with the use of a synthetic boundary cell. The refractive index increment was determined by comparison with bovine serum albumin. This calibration procedure was adopted because the refractive index increments of simple proteins do not vary appreciably and only a small amount of material is required.

Determination of Molecular Weights

The Yphantis high speed equilibrium method (6) gave the best results since a low concentration can be employed and the presence of two components is readily apparent in the analysis of the data.

$$M = [2RT/(1 - \bar{v}_p)\omega^2] d \ln C(r)/dr^2$$

where $C(r)$ is the concentration of protein at distance r from the center of rotation, and is proportional to fringe displacement referred to the region near the meniscus. M was obtained from the slope of plots of the logarithm of fringe displacement versus r^2 . If two components are present, the plot yields two linear regions (as in Fig. 2). The molecular weight of the heavier component was obtained by subtracting the contribution from the lower molecular weight component and replotting the data.

A six-channel centerpiece, channel length 3 mm, was used in the experiments. Centrifugation was performed at speeds ranging from 26,000 to 36,000 rpm for time periods of 24–36 hr. Interferograms were ob-

tained with the Spinco-Rayleigh interference system and were recorded on Kodak type II G plates using a Wratten 77A filter. Fringe displacements were measured with a Nikon optical comparator.

The low speed equilibrium method (short column equilibrium method) was also used (7)

$$M_w = [RT/(1 - \bar{v}_p)\omega^2 r_m C_o] dc/dr$$

where M_w is the weight average molecular weight, dc/dr is the height of the Schlein pattern at the midpoint of the cell, and r_m is the distance from the center of rotation. The initial concentration, C_o , was determined in a synthetic boundary run. Centrifugation was carried out at speeds in the range from 6,000 to 8,000 rpm. The central-pair protein was examined at concentrations of 0.2, 0.5, and 0.7 mg per ml and the data were extrapolated to zero concentration. The six-channel and eight-channel centerpieces were used for both low speed equilibrium and synthetic boundary experiments.

Viscometry

The intrinsic viscosity $[\eta]$ was determined by extrapolation of the specific viscosity obtained at various concentrations to zero concentration.

Measurements were made with a Cannon-Ubbelohde constant volume viscometer with a flow time of 4 min and 35 sec for distilled water. All measurements were made in triplicate at a temperature of $25^\circ \pm 0.05^\circ\text{C}$. The composition of the solution was 6 M guanidine hydrochloride, 0.4 M KCl, 0.2 M mercaptoethanol, 0.01 M tris buffer, pH 8. The protein solution was dialyzed exhaustively against this solution before measurements were made.

Disc Electrophoresis

The reduced-alkylated protein was dialyzed against 8 M urea for 24 hr with frequent changes of the urea solution. Polyacrylamide gels were prepared in 8 M urea, pH 8.7, and electrophoresis was carried out in a Canalco apparatus. Protein was stained with Amido Schwartz and the positions of the bands were determined with a Nikon optical comparator.

Thin-Layer Chromatography

The molecular weight of the colchicine-binding species was determined by thin-layer chromatography on G-200 superfine Sephadex plates. The gel was equilibrated with 0.01 M tris buffer at pH 7.2, and a sample of central-pair protein which had been labeled by incubation with tritiated colchicine was applied. Aliquots of trypsin, pepsin, and bovine serum albumin were run as standards and spotted together with the sample protein. The protein was permitted to migrate for 10 hr at 4°C, and the separated components were transferred to Whatman

3 MM filter paper and air dried. The reference proteins were located by staining with ninhydrin. We then cut the paper into 0.5-cm strips and counted the strips in Bray's solution (8) to determine the location of the bound radioactivity. The molecular weight was calibrated by the method of Andrews (9).

Amino Acid Analysis

Protein samples were centrifuged for 3 hr at 60,000 rpm for removal of any high S contaminants and aggregates, dialyzed for 5 hr against 0.2 M ammonium bicarbonate at pH 8.0, and lyophilized. The samples were hydrolyzed for 24 hr according to the procedure of Moore and Stein (10). Losses due to hydrolysis were estimated at 5% for threonine and tyrosine and 10% for serine.

The amino acids were separated by ion exchange chromatography on a Dowex 50-X8 column in a Technicon automatic amino acid analyzer.

Tryptophan content was determined from the specific absorbance at 2800 and 2944 Å in 0.1 M NaOH (11). Cysteine was determined by titration in 8 M urea with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The absorbance at 4120 Å was measured after 20 min (12).

One sample of central-pair protein, delipidated by the method of Scanu et al. (13) in ethanol-ether, gave essentially the same amino acid composition. Only proteins from *S. purpuratus* were used for amino acid analyses.

Nucleotide Determination and Binding

The presence of bound nucleotide in the outer doublet tubules was determined by precipitation of isolated, intact tubules with 10% trichloroacetic acid (TCA). The protein was then sedimented and the supernatant was extracted with water-saturated diethyl ether to remove the TCA (14). Ultraviolet-absorption spectra were recorded in the Cary spectrophotometer.

Central-pair protein was extracted by a 3-hr dialysis against Tris-EDTA and then precipitated in TCA and studied in the manner described above.

Attempts were made to study the binding of free ATP and GTP to the central-pair and outer-doublet proteins. The proteins were incubated with 10^{-4} M nucleotide for 4 hr at 4°C in the presence of 10^{-3} M Mg^{++} . Free nucleotide was removed by gel filtration on G-100 Sephadex. Both tritiated and cold nucleotides were used, and results were obtained from liquid-scintillation counting and from ultraviolet absorption spectra both before and after the precipitation of protein in TCA.

Colchicine- 3H binding was measured by incubating samples dissolved in 0.01 M phosphate buffer, pH 7, with 2×10^{-5} M colchicine- 3H for 1 hr at 37°C. Bound radioactivity was determined by gel filtration

on G-100 Sephadex columns (1 × 15 cm) as described previously (2). The number of colchicine-binding sites was also determined with a model E ultracentrifuge equipped with a monochromator and scanning ultraviolet-absorption optics. After incubation with colchicine, the solution was centrifuged at 44,000 rpm and scanned at 280 and 350 mμ for protein and colchicine, respectively. The molar absorption coefficient of colchicine at 350 mμ was determined to be 1.63×10^4 liters moles $^{-1}$ cm $^{-1}$. After formation of the protein boundary, the excess optical density at 350 mμ was measured with respect to the meniscus.

The absorption at 350 mμ of protein-bound colchicine was essentially the same as that of free colchicine. The parameters were determined by a comparison of the absorption before and after precipitation of the protein zone from a Sephadex G-100 column separation. The experiments were performed with colchicine-binding protein from brain,¹ since relatively large quantities of protein were required.

The concentration of added colchicine, 6×10^{-5} M, was sufficiently high to saturate the binding site, based on the previous determination of a binding constant of approximately 10^6 M $^{-1}$ (2).

RESULTS

Sedimentation Constant and Molecular Weight

The central-pair protein is readily obtained in essentially pure form by dialysis of cilia against Tris-EDTA. A major problem was the tendency of this material to aggregate at moderate concentrations, particularly in the presence of salt. High S material was removed just prior to the experiment by a 4-hr centrifugation at 60,000 rpm in the Spinco 65 rotor. The extent of aggregation varied in different experiments, but an extrapolated sedimentation constant ($S_{20,w}^0 = 6.2$) was obtained from a few favorable preparations (Fig. 1). We made measurements in 0.1 M KCl to suppress charge interactions.

The molecular weight was determined on preparations which showed essentially a single component by disc electrophoresis after denaturation and alkylation. The Yphantis meniscus depletion method was found to be most suitable since it allows use of very low concentrations and thus reduced the aggregation problem. In addition, the presence of more than one component is detectable by this method. On material examined immediately after isolation, a single component $M = 120,000$ was occasionally found, but in older preparations there were two components present

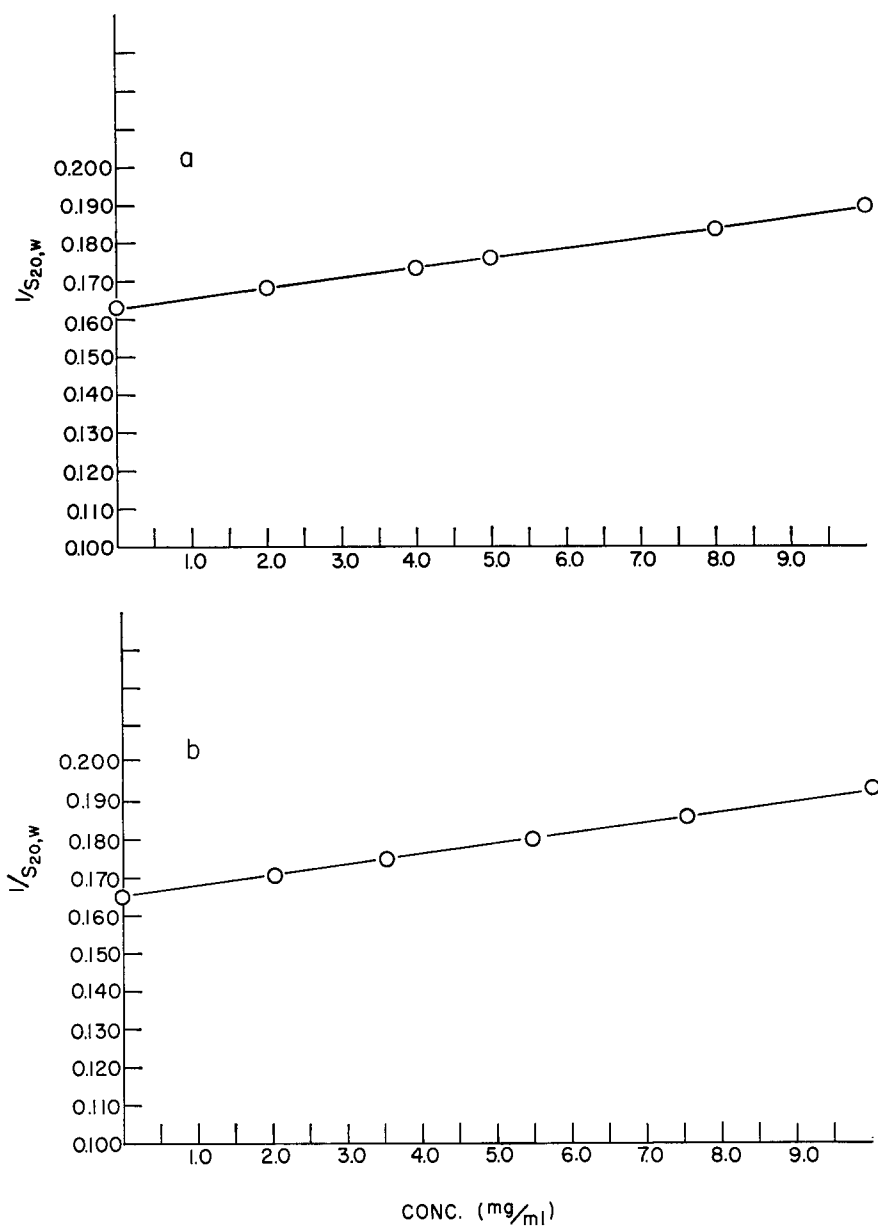


FIGURE 1 Concentration dependence of the reciprocal of the sedimentation coefficient of (a) central-pair protein and (b) outer-doublet protein. All experiments in 0.1 M KCl, 0.01 M phosphate buffer, pH 7.0.

with values of 60,000 and 120,000 (Fig. 2). The presence of two components was the more frequent finding even with fresh material. Reduction and alkylation of preparations containing both components yielded a single component, $M = 60,000$ (Fig. 3).

The values are summarized in Table I. In all

calculations, a partial specific volume of 0.73 was used, obtained from the amino acid composition. The same \bar{v} and no preferential binding of guanine hydrochloride was assumed for molecular weight determinations of denatured protein in guanidine hydrochloride. This assumption was made because not enough protein was available to determine \bar{v} ,

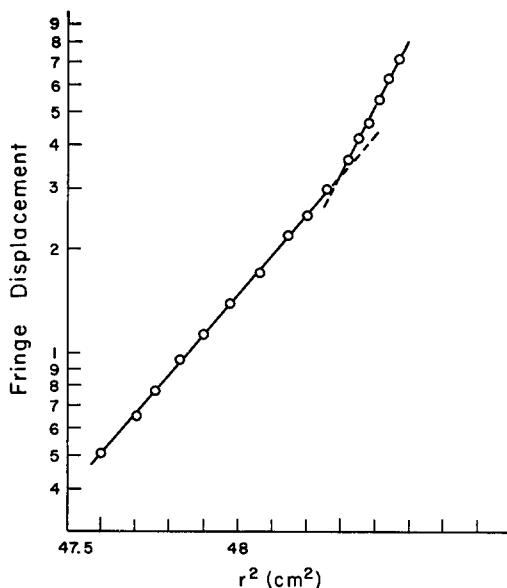


FIGURE 2 Molecular weight determination of native central-pair protein by high speed equilibrium method. Concentration, which is proportional to vertical deflection of the fringes, is plotted versus r^2 , where r is the distance from the center of rotation. Molecular weights were obtained from the slopes of the curves. Two components are present, with molecular weights of 60,000 and 130,000, in this particular experiment. (0.1 M KCl, 0.01 M phosphate buffer, pH 7.5, 20°C)

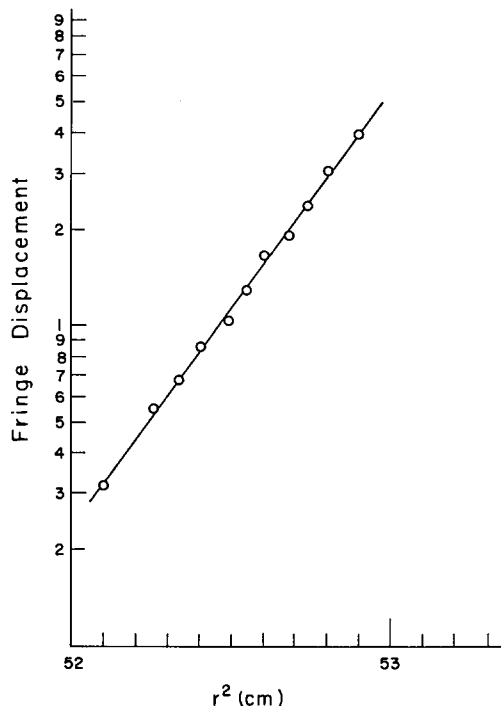


FIGURE 3 Molecular weight determination by high speed equilibrium method of the reduced-alkylated central-pair protein in 6 M guanidine hydrochloride, 0.4 M KCl, 0.01 M tris buffer, pH 8. A single component of molecular weight 60,000 is present.

but the data compiled by Tanford (15) show little change in \bar{v} for most proteins.

The subunit of the outer nine doublets was obtained by dialysis against 10^{-2} M mercaptoethanol in 0.05 M carbonate buffer at pH 10.5. Central-pair protein and dynein were removed by prior extraction in 0.6 M KCl. Although this procedure is harsher than that employed with the central pair, subunit preparations showing a single component of sedimentation constant 6S were sometimes obtained, but formation of aggregates and breakdown to lower S material also occurred. The extracted protein was soluble in 0.1 M KCl at pH 7, and some molecular weight determinations were made in this medium. An extrapolated sedimentation constant of 6.0S was obtained from favorable preparations (Fig. 1). Molecular weight determinations by the low speed equilibrium method gave a value of 118,000. The meniscus depletion method generally showed the presence of two components, $M = 60,000$ and 120,000. Reduction and alkylation in 6 M guanidine again

gave a single component, $M = 60,000$. The two components will be referred to as the monomer and dimer.

Molecular weight estimates were also made for central-pair protein by thin-layer chromatography on Sephadex G-200 Superfine. The plate was calibrated with trypsin, pepsin, and BSA, and the protein was located by the peak of colchicine- ^3H bound to it (Fig. 4). This method yielded a molecular weight of 125,000 and, although not so accurate as the absolute methods, it provides independent evidence that the binding species is the 120,000 component. A faint spot of molecular weight 58,000 was detected, which may have been due to the monomer. No radioactivity was associated with this spot.

It should be noted that in a large number of Sephadex column experiments with colchicine bound to the protein, essentially a single radioactivity peak was found, indicating that binding either to aggregates, which are voided by the

TABLE I
Molecular Weight Determinations on Axonemal Proteins

Source	Preparation	No. of deter- min.	Molecular weight	Method
Sea urchin sperm, Central pairs.	Native	7	120,000 ± 7000	Meniscus depletion
	Native	5	62,000 ± 3000	Meniscus depletion (least component)
	Native	2	124,000 ± 9000	Thin-layer Sephadex
	Denatured	2	61,000 ± 5000	Meniscus depletion
		1	60,000	Low speed equilibrium
	Denatured (2 wk in guanidine hydro- chloride)	2	30,000 ± 100	Meniscus depletion (least component)
Sea urchin sperm, outer doublets	Native	6	118,000 ± 8000	Meniscus depletion Low speed equilibrium
	Native	4	60,000 ± 3000	Meniscus depletion (least component)
	Denatured	2	61,000 ± 1000	Meniscus depletion
		1	57,000	Low speed equilibrium
	Denatured (2 wk in guanidine hydro- chloride)	3	31,000 ± 400	Meniscus depletion (least component)
<i>Tetrahymena</i> cilia, outer doublets	Denatured	1	58,000	Low speed equilibrium

The low speed equilibrium technique was used only in cases in which one component was seen by the meniscus depletion method. The partial specific volume was assumed to be 0.73 for all calculations. "Denatured" refers to proteins in 6 M guanidine hydrochloride, reduced and alkylated as described in text. Molecular weights of native central-pair proteins were determined in 0.1 M KCl, 0.01 M phosphate buffer, pH 7.5. Molecular weights of native outer-doublet proteins were determined in same solution or in bicarbonate extraction solution, pH 10.5, plus 0.1 M KCl.

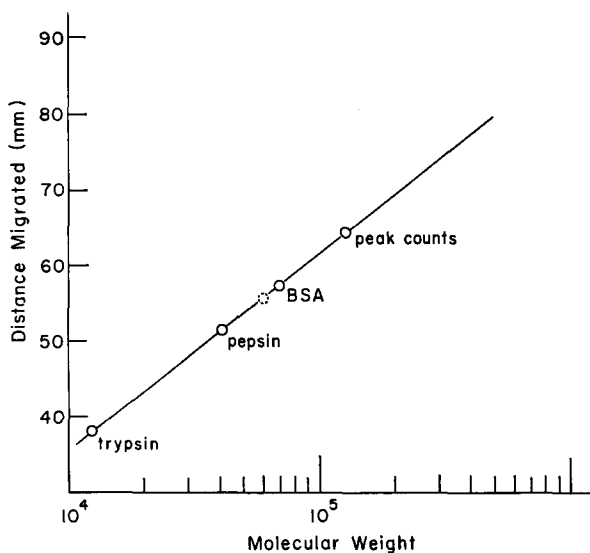


FIGURE 4 Determination of molecular weight of the colchicine binding site by thin-layer chromatography on G-200 Superfine Sephadex. Colchicine-binding protein was incubated with colchicine-³H prior to separation. Radioactivity is present in a single peak of molecular weight 125,000 in this particular experiment. Proteins were located by ninhydrin color reaction. (The dashed circle is a faint protein spot.)

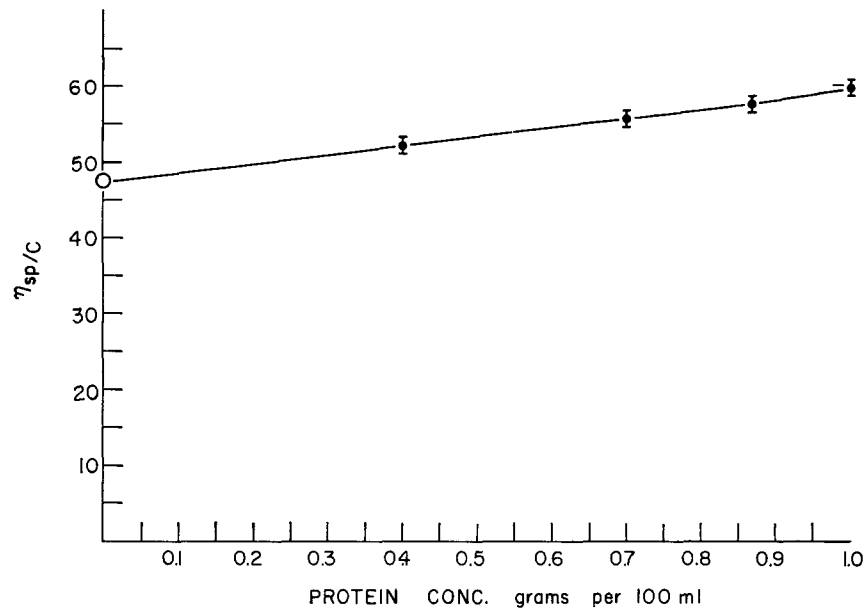


FIGURE 5 Determination of intrinsic viscosity of reduced central-pair protein in 6 M guanidine hydrochloride, 0.4 M KCl, 0.2 M mercaptoethanol (RSH), 0.01 M tris buffer, pH 8.

column, or to a $M = 60,000$ component was not appreciable.

A third estimate of the molecular weight was obtained by measuring the diffusion constant in a synthetic boundary cell. The value of $D_{20w} = 5.2 \times 10^{-7}$ and the extrapolated S value gave a molecular weight of 140,000. This method is less reliable than the meniscus depletion method and more sensitive to higher molecular weight aggregates which would be removed in high-speed equilibrium runs.

Viscometric studies of the monomer obtained from central-pair tubules in guanidine hydrochloride plus 0.2 M mercaptoethanol gave an intrinsic viscosity $[\eta]$ of 47.3 cc/gram (Fig. 5). Using the calibration curve of Tanford (15), this value corresponds to a molecular weight of 63,000, in agreement with the value from sedimentation, and thus indicates that the particle exists as a random coil in this solvent.

For both the central-pair and outer-doublet proteins a third component $M = 30,000$ was obtained after long standing (up to 2 wk) in guanidine hydrochloride. The component was detected by the meniscus depletion method and represented up to 25% of the total protein. Since the material initially consisted of essentially a single component by disc electrophoresis, the result

suggests that the 60,000 molecular weight subunit can break down into two smaller units. This unit has not been characterized and because of the long exposure to guanidine hydrochloride it may be due to nonspecific degradation products with an average weight of 30,000.

A single determination of the monomer molecular weight for the subunit of outer doublets of *Tetrahymena* cilia by the low speed equilibrium method gave a value of 58,000 in guanidine hydrochloride. The protein was prepared by procedure 3 of Renaud et al. (4), and the result confirms the value reported by these authors.

Disc Electrophoresis

The electrophoretic mobilities of central-pair and outer-doublet proteins were compared by the disc electrophoresis technique after denaturation, reduction, and alkylation (Fig. 6). The results are summarized in Table II. Both proteins run separately or as mixtures gave a single major band, $R_f = 0.27-0.28$, plus a number of faint bands. Reduced and alkylated bovine serum albumen was included in many of the experiments for a check of reproducibility and also as insurance that different proteins are resolved by the gel.

Thus, under the conditions of these experiments,

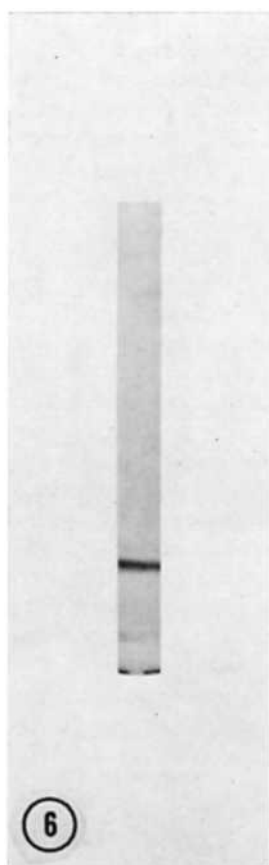


FIGURE 6 Disc electrophoresis pattern of one-to-one mixture of central-pair protein and outer-doublet protein. Reduced-alkylated samples in 8 M urea, electrophoresed at pH 8.7.

namely the reduced-alkylated protein at pH 8.7, the central-pair and outer-doublet subunit proteins consist of a single species of polypeptide chain of identical electrophoretic motility. These experiments do not, of course, rule out the possibility that the monomers are different in their content of uncharged residues. Since we alkylated the proteins to prevent aggregation through disulfide bonds, it is also possible that some charged groups may have been lost in the process, a loss which would reduce the resolution of the method.

Amino Acid Composition

The amino acid compositions of central-pair and outer-doublet proteins from *S. purpuratus* are shown in Table III. It is evident that the two samples show essentially the same amino acid composition.

TABLE II
R_f of Disc Electrophoresis Experiments with Central-Pair and Outer-Doublet Proteins

Source	R _f
Central pair	0.27, 0.27, 0.27
Outer doublet	0.28, 0.27, 0.28
Mixture	0.28, 0.27, 0.27
Bovine serum albumin	0.19, 0.19, 0.21

Each entry refers to a different protein preparation. Reduced-alkylated bovine serum albumin was included as an internal marker in most experiments.

TABLE III
Comparison of the Amino Acid Compositions of the Central-Pair and Outer-Doublet Proteins from S. Purpuratus

Amino acid	Central pairs residues/10 ⁵ g	Outer doublets residues/10 ⁵ g
ASP	85	85
THR	55	54
SER	47	47
GLU	124	123
PRO	46	41
GLY	70	70
ALA	68	71
VAL	56	58
CYS/2	13	13
METH	22	26
ILEU	40	41
LEU	70	67
TYR	27	29
PHE	34	39
LYS	54	51
HIST	25	25
ARG	45	42
TRYP	7	7

Only phenylalanine and proline differ by more than 5%, which is the reproducibility expected for identical samples. As has already been pointed out by Renaud et al. (4), the amino acid composition is very similar to actin.

Binding of Colchicine and Nucleotides

In all of the work done on colchicine binding to microtubule proteins from whatever source, a constant difficulty has been the lability of the colchicine binding site (1-3). Previous work suggested that high concentrations of Mg and ATP or of colchicine itself preserved binding ability, but no treatment was completely satisfactory.

This lability prevented the use of equilibrium dialysis to determine the number of binding sites per dimer. Because of the slow rate of exchange, a reasonably good estimate of the amount of bound colchicine can be obtained from gel filtration on a short G-100 or G-50 Sephadex column. To reduce the loss of activity, we extracted central-pair protein in the presence of colchicine, removed high S material by centrifugation at 65,000 rpm, incubated an aliquot at 37°C with labeled colchicine for 1 hr, and performed gel filtration on a column equilibrated with added colchicine. The procedure gave a value of only 0.5–0.6 mole colchicine/120,000 g. This value is clearly an underestimate, and the method is subject to the criticism that denaturation occurs at 37°C at a rate which competes with the exchange of cold and labeled colchicine (3).

A better method is provided by measuring the binding of colchicine in the scanning ultracentrifuge, by means of the colchicine absorption band at 350 m μ . For these experiments, the protein, isolated in the presence of 6×10^{-5} M colchicine, was incubated at 37°C for 1 hr and immediately centrifuged at 4°C. The loss of bound colchicine at the latter temperature is relatively small in the course of the centrifuge run. The cell was scanned at 280 m μ for protein and 350 m μ for colchicine (Fig. 7). It is evident that a single colchicine-binding species is present. This method yielded values of 0.8 and 0.9 mole colchicine/120,000 g. Unfortunately, there are still errors inherent in the method which will tend to produce a low figure. Some dissociation occurs during the run, as shown by a trail of 350 m μ absorption behind the protein boundary. Some aggregation occurred as evidenced by a small increase in absorption at 280 m μ

centrifugal to the main protein boundary in a region of constant absorption at 350 m μ . A correction was made to the protein concentration for the small amount of aggregated material.

Colchicine binding to the outer-nine protein was very small, but, in view of the method that had to be used for dissolving the protein, little or no binding was expected.

The important finding by Stephens et al. (14) that guanine nucleotides are strongly bound to the outer doublets of *Tetrahymena* cilia and sea urchin sperm tails prompted us to determine whether the central-pair protein also has a guanine nucleotide-binding site. The extent of binding to outer-doublet material was approximately 1 mole per 60,000 g, in agreement with the results of Stephens et al. (14). The bound nucleotide did not give measurable exchange when incubated for 4 hr at 4°C in a solution containing 10^{-4} M tritium-labeled GTP and 10^{-3} M MgCl₂. The presence of bound GTP was also demonstrated with outer doublets from sperm of a marine worm (*Urechis caupo*) and a starfish (obtained in New York area, species unknown). However, attempts to demonstrate guanine nucleotide binding to the central-pair protein were initially unsuccessful. The protein as isolated in 10^{-4} M EDTA did not contain bound nucleotide. Incubation of protein with cold or tritium-labeled ATP or GTP for 4 hr at 4°C in the presence of 10^{-3} M MgCl₂ did not give detectable binding as measured by absorption spectrum or radioactivity, after gel filtration on G-100 Sephadex.

In other studies on the binding of guanine nucleotides to the microtubule protein from mammalian brain,¹ it was found that a concentration of 10^{-2} M MgCl₂ was necessary to retain the nucle-

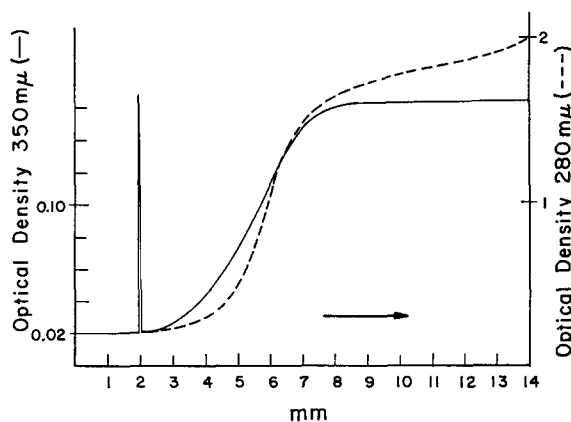


FIGURE 7 Scanning ultracentrifuge trace (redrawn) of colchicine-binding protein centrifuged in presence of colchicine. Pattern was scanned for protein (optical density at 280 m μ) and colchicine (optical density at 350 m μ), 44,000 rpm, 20°C. Solution contains 0.01 M MgCl₂, 0.01 M phosphate buffer, pH 6.7, and 6×10^{-5} M colchicine.

otide and to preserve exchange activity. Preliminary experiments with sperm from starfish and sea urchins showed that bound guanine nucleotide can be detected if central-pair protein is extracted in 0.01 M tris buffer, pH 7.2 containing 10^{-2} M $MgCl_2$ and 10^{-4} M GTP. Separation of protein bound from free guanine nucleotide was carried out on a G-75 Sephadex column. Precipitation of protein with TCA released a guanine nucleotide, as determined by absorption spectrum in the amount of 0.25 mole per 60,000 g. The low value is partly due to dissociation of bound nucleotide during the column separation step.

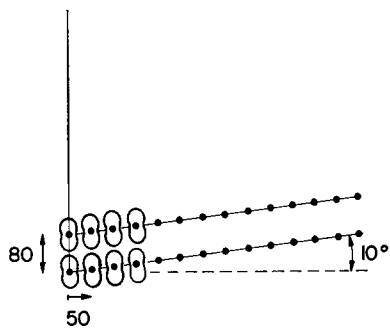


FIGURE 8 Model of microtubule surface lattice. The dimer subunits ($M = 120,000$) are placed on a helix with 13 residues per turn, pitch 10° , and period 80 Å. The subunits are represented by indented figures to indicate that the morphological unit seen in the electron microscope will have a dimension of approximately 40 Å.

CONCLUSIONS

The central-pair and outer-doublet tubules of sperm flagella consist primarily of a single protein subunit which is very similar if not identical for both types of tubules. For each structure, the subunit is obtained as a moderately stable dimer, $M = 120,000$, $S = 6$, which partly dissociates into a monomer form, $M = 60,000$, on standing, or completely dissociates after denaturation in 6 M guanidine hydrochloride and reduction in mercaptoethanol. The two sources yield proteins of the same electrophoretic mobility in the reduced-alkylated form and the same amino acid composition. Both proteins possess a binding site for guanine nucleotide. The central-pair protein also binds colchicine, but colchicine binding has not yet been demonstrated with the outer-doublet protein. This apparent discrepancy is very prob-

ably due to modification of the tertiary structure during the isolation of the outer-doublet protein, since the conditions (overnight dialysis at pH 10.5) lead to a rapid loss of colchicine-binding activity when applied to the protein from central pairs. Also, the ability of the isolated proteins to bind guanine nucleotide was demonstrated only for central-pair protein. Although the outer-doublet tubules contain bound guanine nucleotides, the protein isolated from them did not contain or bind nucleotide, again suggesting that the protein has been obtained in a denatured form.

The molecular weights reported here are similar to those obtained by Renaud et al. (4) for subunits of *Tetrahymena* outer-doublet tubules. Although their values of 104,000 for the material obtained from acetone powders and of 55,000 for the monomer in guanidine hydrochloride are slightly lower these differences are not greater than the experimental errors.

Certain difficulties in the study of the subunit protein must be noted, since they have not been entirely overcome. First, the determination of the physical properties of the protein has been hampered by the tendency of the protein to aggregate, yielding high S material, and of the dimer to dissociate. In the Yphantis high speed equilibrium method, the higher S aggregates will be removed in the course of sedimentation. However, the breakdown of some of the dimer to 60,000 molecular weight components occurred in most molecular weight experiments. Secondly, the ability to bind colchicine was lost rapidly. The protein which had lost much of the colchicine and nucleotide binding activity still had a sedimentation constant of 6S and a molecular weight of 120,000. Colchicine binding was not detected for the high S aggregates.

This instability has prevented an accurate determination of the number of colchicine-binding sites per molecule. There is at least one site per dimer, but experimental errors tend to cause the binding to be underestimated and two sites cannot be completely ruled out.

The similarities between the tubule protein and actin, particularly with regard to amino acid composition, have already been commented upon by Renaud et al. (4). The molecular weight of the monomer is close to that of actin, although more recent determinations of the molecular weight of actin have given values below 50,000 (16). However, a number of properties are distinctly differ-

ent; the microtubule protein is stabilized by guanine nucleotides or colchicine whereas actin is stabilized by adenine nucleotides; the microtubule protein is obtained predominantly as a dimer even when extraction conditions are chosen which would yield monomeric actin; the tubule protein preparation does not polymerize at neutral pH in 0.1 M KCl plus $MgCl_2$; the actin dimer appears to be denatured since it has lost the nucleotide-binding site and the ability to polymerize whereas the tubule dimer shows colchicine and nucleotide binding and we would judge it to be the native form of the protein. The interesting possibility remains that there is an evolutionary relationship between these proteins rather than an identity.

It is important to correlate the chemical results with the structure of microtubules determined by electron microscopy. Although diameters varying from 180 to 350 Å have been reported, there appears to be a large class of microtubules with a diameter of approximately 240 Å (17), consisting of 13 parallel subfilaments each made up of subunits 40–50 Å in diameter. In some cases, the surface lattice has been partly resolved and the row of subunits is seen to make an angle of about 10° to the plane perpendicular to the tubule axis. A cross-banding pattern with a period of 80 Å has also been described (18, 19). Thus, the structure can be represented by 13 parallel filaments with a displacement between the subunits of neighboring filaments or by a helix with 13 residues per turn.

The size and shape of the chemical subunit can be estimated from the molecular weight, sedimentation, and diffusion constants. Since a 50-Å spherical particle of $\bar{v} = 0.73$ has a molecular weight of only 50,000, the dimer is too large to be identified with the morphological subunit. The parameter f/f_m , which is the ratio of the actual frictional coefficient to that of an anhydrous sphere of the same molecular weight (20), is 1.37. In order to be spherical, the dimer would require a hydration of more than 1 g water per g protein, which is unreasonable. If the value of 0.3 g per gm is taken as fairly typical of proteins, the axial ratio of the equivalent hydrodynamic ellipsoid is 4 to 5. The use of an ellipsoid as a hydrodynamic model tends to overestimate the axial ratio compared to a more rodlike or brick-shaped particle. Allowing for errors in the experimental parameters, the data are probably consistent with a

fairly compact shape, of axial ratio 2.0 to 3.0; for example, a rod $45 \times 45 \times 90$ Å or a brick $40 \times 40 \times 90$ Å would give the required molecular weight. Since hydrodynamic parameters are not available for shapes other than ellipsoids, comparison cannot be pursued further, but it is fairly clear that the physical evidence requires that the dimer corresponds to two of the 40-Å morphological subunits. Renaud et al. (4) have also concluded that the monomer is the 40-Å unit. The only other plausible model is to approximate the shape of the dimer by a row of four 40-Å spheres. However, this structure is not consistent with the electron microscope evidence discussed in the next paragraph or with the number of colchicine and GTP-binding sites.

The simplest model which gives the best overall agreement with the chemical and electron microscopic evidence is illustrated in Fig. 8; the tubule is presented as it would appear if cut parallel to its axis and rolled out flat. The dimer of dimensions 40–50 Å by 80–90 Å has been oriented with its long axis parallel to the tubule axis. A transverse orientation is ruled out by a wall thickness of only 50 Å. Placement of the long axis tangential to the tubule would not account for the apparent division of the tubule into parallel filaments. The filaments also appear to be a stable packing of subunits since single filaments can be seen splaying out from damaged tubules, an unlikely result if the dimer is shared by adjacent filaments. The repeating unit is thus a helix with 13 dimers per turn, and a displacement of 80–90 Å. These dimensions are consistent with the 10° pitch and the apparent cross-banding pattern with spacing of 80 Å seen in the electron microscope.

Although this description allows a correlation to be made between the chemical and morphological evidence, it is at best a first approximation to the structure since several features are unexplained. The subunits of central-pair and outer-doublet tubules (and other cytoplasmic microtubules) appear to be identical, yet the arms are found only on the A subfiber of the outer doublets, and in only two positions. Furthermore, the arms are arranged with a longitudinal period of about 150 Å. The important work of Grimstone and Klug (21) showed the presence of spacings of 160, 80, 53, and 40 Å in the optical diffraction patterns of cilia microtubules, i.e. the first four orders in the diffraction of a structural unit with

a longitudinal spacing of 160 Å. These authors point out that the surface lattice may be perturbed by a zig-zag pattern of the morphological subunits along a filament, thus accounting for the 80-Å spacing and for the six-sided figures sometimes visible on the surface of tubules. An explanation of the 160-Å period was not given.

Since the present work favors 80 Å as the true subunit repeat, it is necessary to reconsider the evidence from optical diffraction. The resolution of the optical diffraction pattern would not be sufficient to distinguish a linear array of monomers from an array of dimers if the dimer is constructed from identical subunits and is oriented parallel to the tubule axis. Thus, the occurrence of 80- and 160-Å reflections would still require a zig-zag configuration along a subfilament, except that the 160-Å period could be explained simply by a perturbation of the dimers rather than the monomers.

Finally, it may be noted that the dimer in solution readily loses the colchicine- and GTP-binding site without change in molecular weight and with little or no change in sedimentation constant. As will be described in detail elsewhere, GTP stabilizes the molecule. Thus, the dimer may exist in two or more configurations which depend in

some way on GTP binding. It is relevant to ask whether a deformation of the surface lattice plays a functional role in cilia movement. The conversion of a straight subfilament to a zig-zag pattern in the plane radial to the surface, brought about by a configuration change of the dimer unit, would serve to bend the tubule. The deformation could be initiated (or reversed) by hydrolysis (or phosphorylation) of bound nucleotide. At this stage, a detailed hypothesis is hardly worth advancing, but a study of the configuration and interactions of the subunit protein would appear to provide a rational approach to the question of the molecular mechanism.

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