CHLAMYDOMONAS FLAGELLA

II. The Distribution of Tubulins 1 and 2 in the Outer Doublet Microtubules

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ABSTRACT

Quantitative ultrastructural analysis and quantitative gel electrophoresis of preparations of selectively solubilized *Chlamydomonas* outer doublets indicated that tubulins 1 and 2 were present in both the A tubule and the B tubule, and that only tubulin 1 was present in the three protofilaments which form the wall ("partition") between the lumens of the A and B tubules. The data suggested that the remaining protofilaments of the outer doublet were grouped together in pairs containing the same type of tubulin, pairs containing tubulin 1 alternating with pairs containing tubulin 2. These findings were used to construct models for the arrangement of the two tubulins in the outer doublet. Further analysis by isoelectric focusing resolved tubulins 1 and 2 into at least five bands.

INTRODUCTION

The fine structure of the outer doublet microtubules of cilia and flagella has been the subject of numerous investigations (e g., André and Thiéry, 1963; Pease, 1963; Grimstone and Klug, 1966, Barton, 1969; Thomas, 1970). One of the most intensive of these was the study of the outer doublets of *Chlamydomonas* by Ringo (1967), who proposed a model for the number and arrangement of the protofilaments (or subunits as observed in cross-section) of the outer doublet microtubules. In this model the outer doublet contains a total of 23 protofilaments, arranged as illustrated in Fig. 1: The A tubule of the outer doublet contains 13 protofilaments, three of which are located medially and form the "partition" between the lumens of the A and B tubules; the B tubule contains 10 protofilaments. Each protofilament is made up of a single row of spherical subunits. These features of the outer doublet were determined by Ringo from (*a*) inspection of high-resolution electron micrographs of longitudinal and cross-sections of outer doublets; (*b*) Markham rotational reinforcement analysis of electron micrographs of cross-sections of outer doublets; (*c*) measurement of the diameter and center-to-center spacing of the protofilaments; and (*d*) comparison of actual electron micrographs of thin sections of outer doublets with a number of model configurations for the arrangement of the protofilaments in the outer doublet.

In the preceding report (Witman et al , 1972) methods were described for the isolation and fractionation of the outer doublet microtubules of *Chlamydomonas*. Electrophoretic analyses of the isolated intact outer doublets indicated that they contained two microtubule proteins or "tubulins" (Mohri, 1968); these proteins (called tubulins 1 and 2 here) have been separated and purified, and have been shown to have different amino acid con-

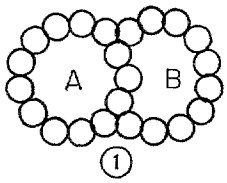


FIGURE 1 Diagram illustrating the arrangement of the subunits (protofilaments) in an outer doublet of *Chlamydomonas* as determined by Ringo (1967).

tents and molecular weights (Olmsted et al, 1971, Carlson, Witman, and Rosenbaum, in preparation). In the experiments described in this report, outer doublets in various stages of solubilization were analyzed by both quantitative acrylamide gel electrophoresis and by quantitation of electron micrographs to determine the distribution and arrangement of the two tubulins relative to the fine structure of the outer doublet as described by Ringo. The results indicated that (*a*) both tubulins were present in the A tubule and the B tubule, (*b*) only tubulin 1 was present in that portion of the A tubule which forms the partition between the lumens of the A and B tubules, and (*c*) of several models which could be constructed for the arrangement of the tubulins in the remainder of the outer doublet, one in which *pairs* of protofilaments containing only tubulin 1 alternated with *pairs* containing tubulin 2 fit the experimental data quite well.

MATERIALS AND METHODS

Culture of Chlamydomonas and Isolation of Flagella

The methods used for culturing and harvesting wild-type 21-gr ("9 + 2") and mutant pf-18 ("9 + 0") *Chlamydomonas* and for detaching and isolating the flagella were described in the preceding report (Witman et al., 1972).

Fractionation of Flagella

Preparations of intact outer doublets were obtained from "9 + 2" flagella of wild-type cells or from "9 + 0" flagella of mutant cells by the procedures previously described (Witman et al., 1972).

Quantitative Electron Microscope Analysis of Outer Doublets

Pellets containing outer doublets or parts thereof were fixed, flat-embedded, and sectioned so that large numbers of cross-sections of outer doublets were obtained, all as described in the preceding report (Witman et al., 1972) Considerable care was taken to section the complete axis of the pellet from top to bottom; in addition, a number of other regions of the pellet were also sectioned. Electron micrographs of the sections were taken at a sufficiently high magnification to permit quantitating the number of subunits (i.e. protofilaments) which appeared in a cross-section of an outer doublet. This was done by projecting the electron microscope plates directly onto a screen and counting the subunits in the A and B tubule. As described in detail in the previous report (Witman et al, 1972), the A tubules could be easily distinguished from the B tubules because of their shape and position, and the inner and outer portions of each tubule could be differentiated because the outer doublets tended to maintain their circular arrangement during solubilization.

Quantitative Electrophoretic Analysis of Outer Doublets

The outer doublet preparations were analyzed by electrophoresis in urea acrylamide gels as previously described (Witman et al., 1972). These gels were stained with fast green and then traced with a Joyce-Loebl microdensitometer (Gorovsky et al., 1970). The relative amounts of tubulins 1 and 2 in the preparations were determined by integrating the appropriate peaks of the scan with a planimeter, or by cutting out the peaks and weighing them. For each preparation two or sometimes three gels were analyzed and the amount of protein in the tubulin 1 and 2 peaks agreed to an accuracy of $\pm 1\%$. In the gel system used, fast green stains microtubule proteins in amounts up to 150–200 μg/gel with no appreciable deviation from linearity (Gorovsky et al., 1970); the micrograms of protein applied to each gel were kept well below this amount.

Determination of Protein Concentration

Before electrophoresis, the concentration of protein in each sample was determined by the procedure of Lowry et al. (1951).

RESULTS

(I) *Distribution of Tubulins 1 and 2 in the Outer Doublet*

In the preceding report (Witman et al., 1972) it was demonstrated by electrophoretic analysis that the isolated outer doublets of *Chlamydomonas* flagella contained two tubulins. Although it had previously been reported by Stephens (1970) and

TABLE I

Quantitative Analysis of Tubule and Tubulin Content of Outer Doublet Preparations

Preparation	Electron microscope analysis		Electrophoretic analysis	
	Per cent of total protofilaments found in the B tubule	Per cent of total protofilaments found in the A tubule	Per cent of total microtubule protein in Tubulin 1	Per cent of total microtubule protein in Tubulin 2
Intact outer doublets (see Fig. 2)	40.9	59 1	50	50
A tubules (see Fig. 3)	5.8	94.2	54	46
B tubules (solubilized)	—	—	48	52

Jacobs and McVittie (1970) that one tubulin was contained solely in the A tubule and the other in the B tubule, analysis of preparations of isolated outer doublets by quantitation of electron micrographs and quantitative gel electrophoresis indicated that the two tubulins actually occurred together in each tubule. For example, in a typical preparation of outer doublets (Figs. 2A and 2B), in which 40.9% of the protofilaments were in the B tubule and 59.1% were in the A tubule[1], the two tubulins were present in a 50:50 ratio, rather than in the 41:59 ratio expected if each tubule contained only a single tubulin (Table I, line 1). This result excluded the possibility that one tubule was composed solely of tubulin 1 and the other of tubulin 2, and suggested that both proteins were present in one or both tubules

Direct evidence that the two tubulins occurred together in both tubules was obtained by electrophoretic analysis of preparations containing predominantly A tubules (Fig. 3A), prepared by treating isolated outer doublets with 0.3% Sarkosyl. These preparations contained both tubulins in nearly equal quantities (Fig. 3B). For example, in a preparation in which 94.2% of the protofilaments were in A tubules and only 5.8% were in B tubules, 54% of the total microtubule protein in the preparation was tubulin 1 and 46% was tubulin 2 (Table I, line 2). These results indicated that *the A tubule of the outer doublet contained both tubulins 1 and 2.*

Furthermore, the relative amounts of the two tubulins in preparations of A tubules were nearly the same as in preparations of intact outer doublets (compare lines 1 and 2 of Table I). This observation indicated that *the B tubule also contained both tubulins.* If the B tubule had contained only one tubulin, then preparations of A tubules would have contained much less of that tubulin than preparations of intact outer doublets.

Electrophoretic analysis of the B tubule proteins solubilized during the preparation of A tubules also indicated that the B tubule contained both tubulins 1 and 2 (Table I, line 3).

In summary, the above results indicated that *both* the A and B tubule of the outer doublet contained tubulins 1 and 2.

(II) *Arrangement of Tubulins 1 and 2 in the Outer Doublet*

To determine where in each tubule the two tubulins were located (i.e , which tubulins were present in each protofilament) preparations of partially solubilized outer doublets containing varying numbers of the different protofilaments were analyzed by quantitative gel electrophoresis The partition (that portion of the A tubule which forms the wall between the lumens of the A and B tubules) could be obtained pure so that its tubulin composition could be determined directly by electrophoretic analysis. Determination of the tubulin composition of the other portions of the outer doublet, which could not be obtained in such purity, required additional data from detailed electron microscope analysis of preparations containing these portions

[1] According to Ringo's model for the structure of the outer doublet of *Chlamydomonas*, 10 of the 23 protofilaments of the outer doublet are located in the B tubule. Therefore, in an ideal preparation of *completely intact* outer doublets, $10/23 \times 100\% = 43.5\%$ of the protofilaments would be in the B tubule and 56.5% would be in the A tubule. However, in actual preparations, a small portion of the B tubule was always missing from some of the outer doublets, while the A tubule remained almost completely intact. Therefore, the per cent of the total protofilaments in the B tubule was always slightly less than in the ideal preparation, and the per cent in the A tubule was always slightly more.

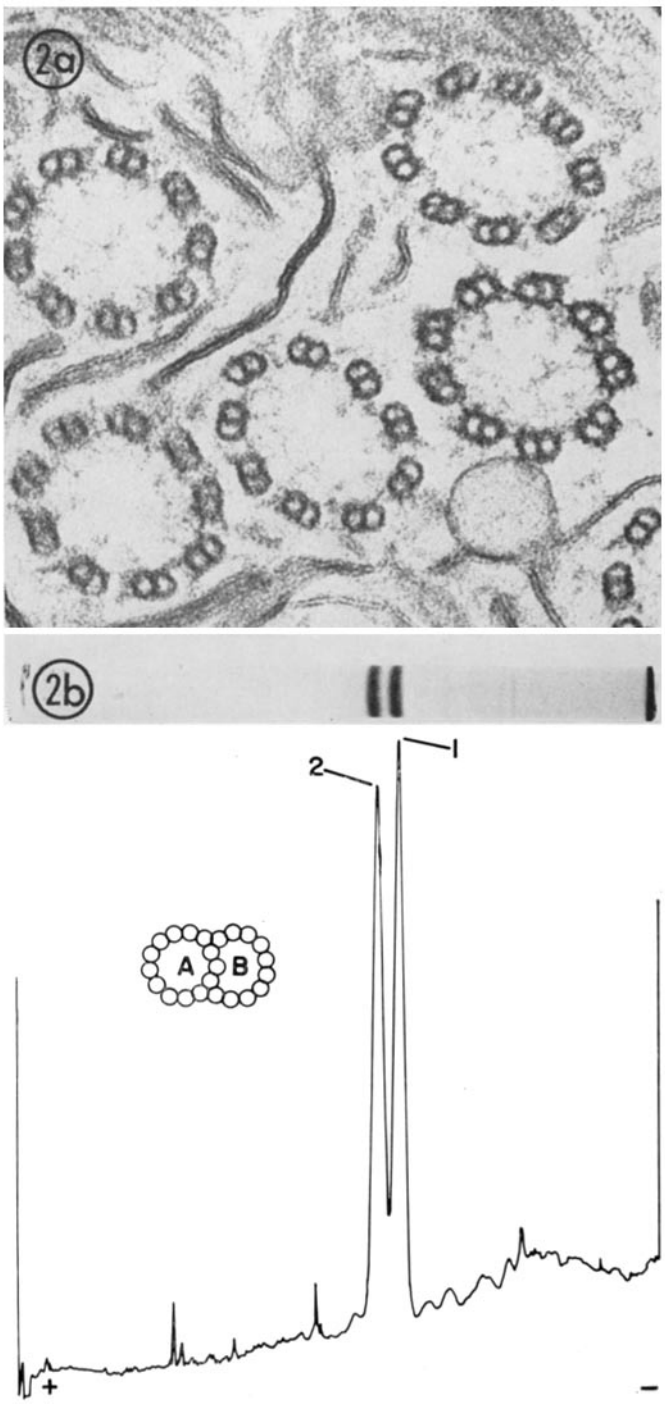


FIGURE 2 Isolated intact outer doublets of *Chlamydomonas* flagella. Fig. 2 a, electron micrograph. × 155,000. Fig. 2 b, urea acrylamide gel and densitometric tracing of outer doublet protein.

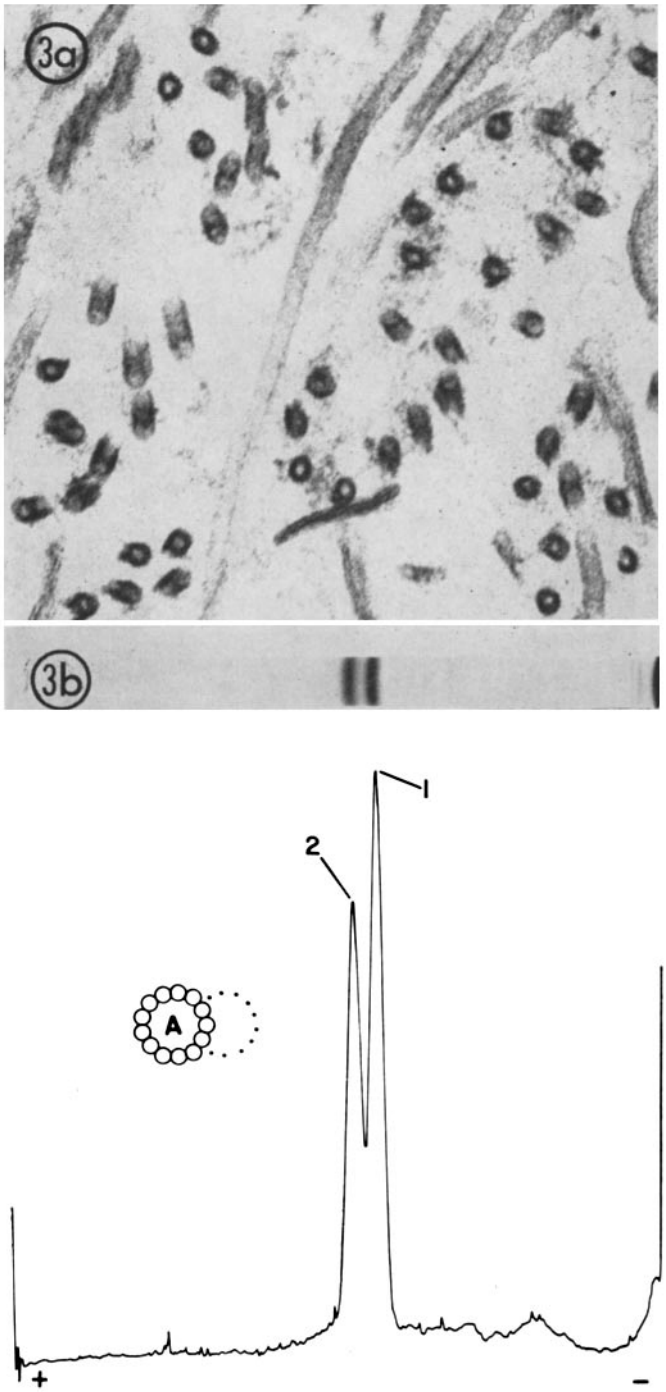


FIGURE 3 Isolated intact A tubules of *Chlamydomonas* outer doublets. Fig. 3 a, electron micrograph. × 155,000. Fig. 3 b, urea acrylamide gel and densitometric tracing of A tubule protein.

(A) Localization of Tubulins in the Partition Protofilaments

Homogeneous preparations of partitions were obtained by treating isolated outer doublets with 0.7% Sarkosyl Each partition was composed of three protofilaments, there were no other protofilaments in the preparations (see Fig. 17 in the preceding report, Witman et al , 1972). Analysis of these preparations by urea acrylamide gel electrophoresis (Fig. 4) indicated that *the three partition protofilaments contained only tubulin 1*

This result revealed two important features of outer doublet microtubule construction. (*a*) At least some individual protofilaments of the outer doublet were made up entirely of *one* tubulin; (*b*) protofilaments containing the same tubulin were grouped together in the outer doublet.

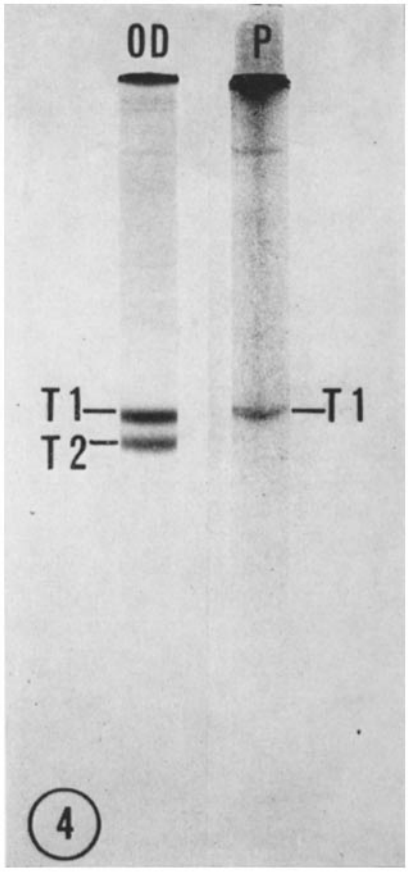


FIGURE 4 Fast green-stained urea acrylamide gels of isolated, outer doublets (*OD*), and of isolated partitions (*P*). The densely-staining material at the origin of the (*P*) gel is due to the presence of membrane contamination in this fraction. Membrane proteins do not migrate in the urea acrylamide gels.

(B) Localization of Tubulins in the Nonpartition Protofilaments

The localization of tubulin 1 in the three partition protofilaments was straightforward because preparations of these protofilaments were free of all other protofilaments, the preparations also contained only one tubulin, so there was no ambiguity as to which protofilament contained which tubulin. It was more difficult to determine the tubulin composition of the other protofilaments of the outer doublet, because preparations of partially solubilized outer doublets containing these protofilaments (*a*) were more heterogeneous than preparations containing only partitions, (*b*) contained many more protofilaments to be taken into account, and (*c*) always contained both tubulins

In order to determine the tubulin composition of each nonpartition protofilament, it was first necessary to determine by quantitation of electron micrographs the relative amounts of the different protofilaments remaining in a preparation of partially solubilized outer doublets Each of the 23 protofilaments (or subunits as viewed in cross-section) of the outer doublet was assigned a number, as illustrated in Fig. 5. Then, cross-sections of at least 500 outer doublets or parts thereof in a preparation of partially-solubilized outer doublets were analyzed to determine whether protofilament 1, 2, 3, . . 23 was still present. The number of subunits remaining in a partially-solubilized outer doublet could be determined to an accuracy of $\pm\frac{1}{2}$ subunit. Quantitation by this procedure is illustrated in Fig. 6 where the outer portions of the B tubules in two outer doublets have been solubilized to different degrees Fig 7 indicates the number of times that a given protofilament was observed at the different stages of solubilization.

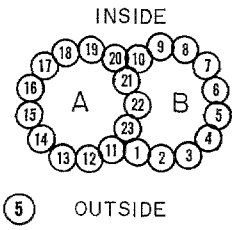


FIGURE 5 Diagram illustrating the assignment of numbers to the 23 protofilaments of the outer doublet. The protofilaments of the B tubule were numbered 1–10, and those of the A tubule 11–23.

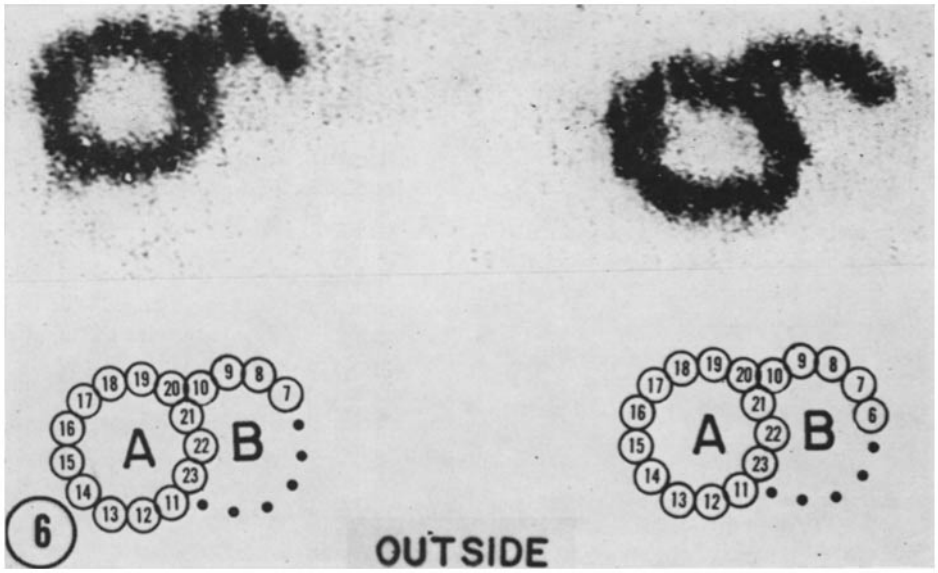


FIGURE 6 Partial solubilization of the outer portions of the B tubules of two outer doublets. The numbering of the protofilaments is shown below each doublet. The doublet on the left has one more subunit in the B tubule than the one on the right. × ~ 1,000,000.

From this data, it was possible to calculate what per cent of the total microtubule protein in each preparation was contributed by the various protofilaments. For example, in a preparation obtained by treatment of outer doublets with 0.3% Sarkosyl, a total of 6108 protofilaments were still present in the 500 outer doublets assayed (Fig. 7 and Table II). In these 500 outer doublets, protofilament 1, observed 104 times, made up 104/6108 × 100% = 1 7% of the protofilaments observed, and consequently contributed 1.7% of the total microtubule protein in the preparation (Table II) In this way, the per cents of the total microtubule protein contributed by each of the protofilaments in this preparation were calculated and are recorded in Table II.

From the per cent of the total microtubule protein contributed by each protofilament in a preparation of partially-solubilized outer doublets, it was possible to determine whether a particular hypothetical arrangement of tubulins in the outer doublet would result in the same relative amounts of tubulin 1 and 2 as found in that preparation by acrylamide gel electrophoresis. For each preparation, the relative amounts of tubulins 1 and 2 predicted from any hypothetical arrangement could be determined by calculating what per cent of the total microtubule protein in that preparation would be contributed by those protofilaments proposed to contain tubulin 1 or tubulin 2. For example, in the arrangement diagrammed in Fig 8, protofilaments 3, 4, 7, 8 . 23 are proposed to contain tubulin 1, while protofilaments 1, 2, 5, 6 . 20 contain tubulin 2 Quantitative analysis of electron micrographs of preparations of partially-dissolved outer doublets resulting from treatment with 0 3% Sarkosyl indicated that protofilaments 3, 4, 7, 8 . . . 23 contributed 53 5% of the total microtubule protein, while protofilaments 1, 2, 5, 6 . . 20 contributed 46.3% (see Table III). Therefore, if the tubulins were arranged as illustrated in Fig. 8, 53.5% of the total microtubule protein in this preparation would be tubulin 1 and 46 3% would be tubulin 2. In this case, these predicted values are in good agreement with the actual values of 54% for tubulin 1 and 46% for tubulin 2 determined by gel electrophoresis.

For several preparations of outer doublets in different stages of solubilization, similar calculations were made for many possible arrangements of the tubulins. Four of these arrangements and their predicted values for the per cents of tubulin 1 in some representative preparations are given in Fig. 9, along with the actual per cent of tubulin 1 found in each preparation by gel electrophoresis For most preparations, the values predicted by arrangement A were in closest agreement with the actual values. The various features of this arrange-

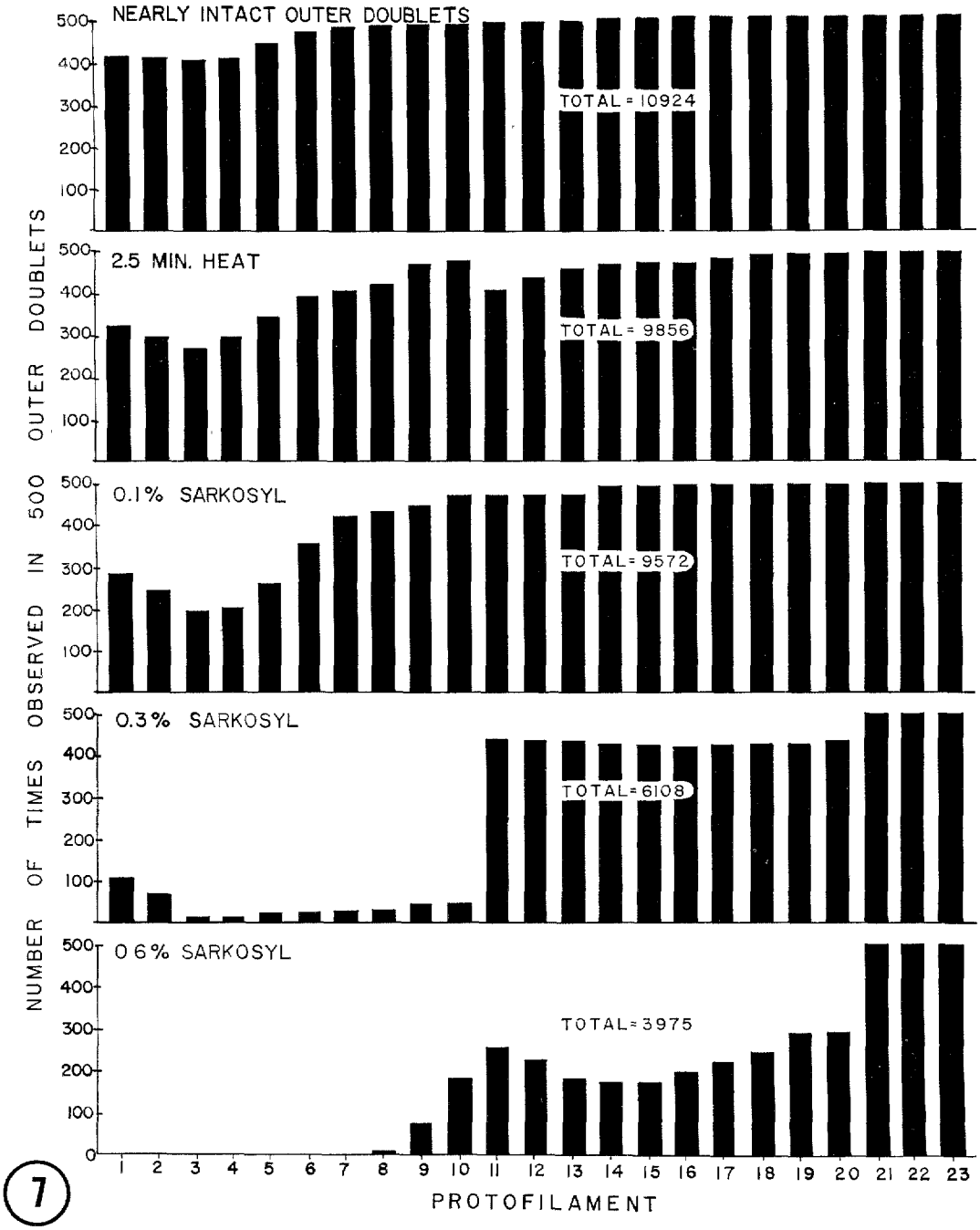


FIGURE 7 The number of times each of the 23 protofilaments of the outer doublet was observed in 500 outer doublets as assayed in preparations of nearly intact outer doublets, or of outer doublets partially solubilized by treatment with heat (46°C for 2.5 min) or with 0.1%, 0.3%, or 0.6% Sarkosyl. The total number of protofilaments observed in the 500 outer doublets assayed in each preparation is also recorded.

TABLE II

Per cent of Total Microtubule Protein Contributed by Each Protofilament in a Preparation Obtained by Treatment with 0.3% Sarkosyl

Protofilament	Number of times each protofilament was observed in 500 outer doublets*	Per cent of total microtubule protein contributed by each protofilament
1	104	1.7
2	63	1.0
3	11	0.2
4	11	0.2
5	19	0 3
6	21	0 3
7	25	0.4
8	27	0 4
9	35	0.6
10	39	0 6
11	436	7.1
12	433	7.1
13	432	7.1
14	423	6.9
15	422	6.9
16	417	6.8
17	418	6 8
18	421	6.9
19	423	6.9
20	428	7.0
21	500	8.2
22	500	8.2
23	500	8.2
Total protofilaments observed in 500 outer doublets	= 6108	99.8

* From Fig. 7.

ment and its relation to previous work are discussed below.

DISCUSSION

Distribution of Tubulins 1 and 2 in the A and B Tubules of Outer Doublets

Quantitative electrophoretic and electron microscope analysis of preparations containing outer doublets, A tubules, or solubilized proteins of the B tubule, clearly indicated that two major microtubule proteins or tubulins were contained in *both* the A and B tubules of the outer doublets of *Chlamydomonas* flagella.

These results are contradictory to those of Stephens (1970), who reported that the A tubule was composed of one protein ("A-tubulin") and the B tubule of another ("B-tubulin") in outer doublets of sea urchin sperm flagella. This disagreement may arise in part from the fact that Stephens was unable to clearly resolve the two sea urchin tubulins with his gel electrophoresis system. More recently, when Stephens' "A-tubulin" and "B-tubulin" were analyzed by electrophoresis on SDS-urea acrylamide gels by Feit et al (1971), each was found to contain two proteins, and the two proteins of "A-tubulin" were shown to have electrophoretic mobilities identical to the two corresponding proteins of "B-tubulin". Furthermore, Meza et al. (1971) have reported that when outer doublets of sperm flagella of another sea urchin were sequentially solubilized, two electrophoretically separable tubulins were observed in all fractions. These recent reports indicate that, as in *Chlamydomonas* flagella, two tubulins occur in both the A and B tubules of the outer doublets of sea urchin sperm flagella.

The findings presented here are also contradictory to those of Jacobs and McVittie (1970), who concluded that the subunits of the A tubules of the outer doublets of *Chlamydomonas* flagella consisted of one protein and those of the B tubule of a different protein. Examination of their data reveals that they found both proteins in fractions enriched for the B tubule, a result similar to those reported in this paper. However, they claim they observed only a single band when preparations of A tubules were analyzed by electrophoresis. The reason for the discrepancy between this result and the findings reported here is not clear; attempts to reproduce their results using their fractionation techniques in this laboratory were unsuccessful. A possible source of the difference is the manner in which Jacobs and McVittie prepared proteins for electrophoresis: they did not routinely alkylate the proteins after reduction; unprotected sulfhydryl groups are known to become oxidized during electrophoresis in 8 M urea, giving rise to artifacts (Smithies et al., 1966) Another possible explanation for their observation is that those preparations which supposedly contained A tubules and yielded a single band may have also contained large numbers of undetected partition protofilaments, which were shown in the present report to be composed of only tubulin 1 (their "α-polypeptide"); the presence of many of these protofilaments would result in the preparation being greatly enriched for that tubulin

TABLE III

Protofilaments. Proposed Tubulin Composition and Contribution to the Total Microtubule Protein in the 0.3% Sarkosyl Preparation

Proto-filament	Per cent of total microtubule protein contributed by each protofilament (from Table II)	Type of tubulin hypothesized to be in each protofilament (see Fig 8)	Per cent of total microtubule protein contributed by each protofilament which is proposed to contain:	
			Tubulin 1	Tubulin 2
1	1 7	2	—	1 7
2	1 0	2	—	1 0
3	0 2	1	0.2	—
4	0 2	1	0.2	—
5	0 3	2	—	0.3
6	0 3	2	—	0.3
7	0.4	1	0.4	—
8	0 4	1	0.4	—
9	0 6	2	—	0.6
10	0 6	2	—	0.6
11	7 1	2	—	7.1
12	7.1	2	—	7.1
13	7 1	1	7.1	—
14	6 9	1	6.9	—
15	6.9	2	—	6.9
16	6.8	2	—	6.8
17	6.8	1	6.8	—
18	6.9	1	6.9	—
19	6.9	2	—	6.9
20	7.0	2	—	7.0
21	8.2	1	8.2	—
22	8.2	1	8.2	—
23	8.2	1	8 2	—
	99.8		53.5	46 3

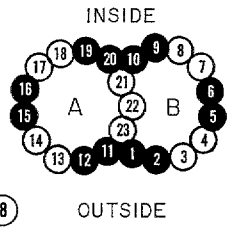


FIGURE 8 Diagram illustrating a hypothetical arrangement of tubulin 1 (white subunits) and 2 (black subunits) in the outer doublet.

The results reported in this paper show the presence of two *electrophoretically similar tubulins in both the A and B tubules* and are, therefore, difficult to reconcile with results from Stephens' laboratory showing that the protein composing the A tubule has a significantly different peptide map from the protein of the B tubule (Stephens, 1970). Moreover, the differential solubilities of parts of the A and B tubules as described in the previous report (Witman et al, 1972) and by others (Behnke and Forer, 1967), also suggests that the A and B tubules are *not* composed of the same two proteins. The results which indicate differences between the proteins of the A and B tubules (peptide mapping, differential solubilities) and those which suggest similarities (electrophoresis) might be explained if tubulins 1 and 2 actually represent *classes* of tubulins, each being composed of two or more proteins whose differences are not great enough to permit their separation on urea or SDS-urea acrylamide gels. That this is probably the case is shown later in this Discussion where results on the isoelectric focusing of tubulins 1 and 2 are presented. By use of this technique, the two tubulins can be separated into at least five bands, and the partition protofilaments which form part of the A tubule focus as only *one* of these bands. If the bands observed on isoelectric focusing represent separate polypeptide chains, then the presence of the partition proto-

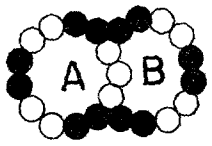
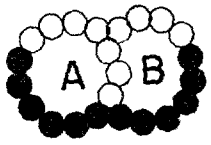
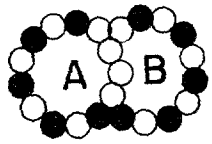
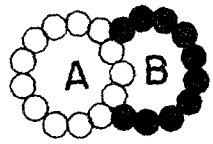
PREPARATION →	NEARLY INTACT OUTER DOUBLETS	2.5 MIN HEAT	0.1% SARKOSYL	0.3% SARKOSYL	0.6% SARKOSYL
ACTUAL PERCENT DETERMINED BY GEL ELECTROPHORESIS →	50%	49%	50%	54%	59%
PERCENT PREDICTED BY MODEL					
A. 	48.1	48.5	48.5	53.5	58.5
B. 	46.5	45.4	44.5	55.7	58 6
C. 	56.7	57.2	57.3	62.3	67.5
D. 	58.5	63.3	65.0	93.3	93.3

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FIGURE 9 Comparison of the per cents of tubulin 1 actually contained in various preparations (as determined by quantitative gel electrophoresis) with the per cents predicted (on the basis of quantitation of electron micrographs) if the tubulins were arranged in the outer doublet as illustrated in the four models (A–D).

filaments in the A tubule would be sufficient to confer differences on the peptide maps of the A and B tubules On the other hand, one must also consider the possibility that the differences in peptide maps between the A and B tubule protein as observed by Stephens (1970) were due to the presence of extramicrotubular structures, e g radial links, which remained attached to the A tubule even after solubilization of the B tubule (Witman, Kuczmarksi, and Rosenbaum, unpublished results).

Location of Tubulins 1 and 2 within the A and B Tubules

Electrophoretic analysis of isolated partitions indicated that these three protofilaments contained only tubulin 1. The tubulins contained in each of the other protofilaments could not be identified with as much certainty Comparisons of the relative amounts of the tubulins found in preparations of partially-dissolved outer doublets by quantitative electrophoretic analysis with the amounts predicted by various hypothetical arrangements indicated that the most likely arrangement for the two tubulins in the outer doublet is the one shown in Fig 9 (A). However, this arrangement is only tentative, since other arrangements, such as that diagrammed in Fig 9 (B), resulted in almost as good an agreement between the real and predicted values.

A three-dimensional model for the outer doublet based on the arrangement in Fig 9 (A) is illus-

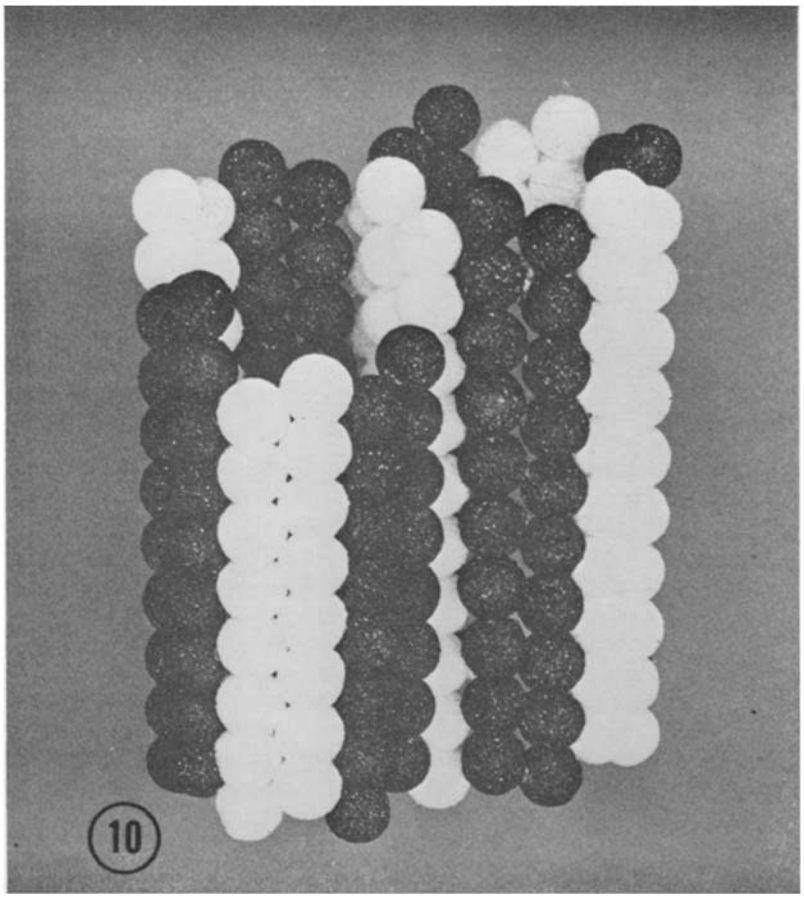

FIGURE 10 Model for the arrangement of tubulins 1 (white subunits) and 2 (black subunits) in the A tubule (right) and B tubule (left) of the outer doublet.

trated in Fig. 10 In this model, the A tubule is composed of 13 protofilaments and the B tubule of 10 protofilaments, as indicated by the ultrastructural analyses of Ringo (1967) [2] Alternate protofilaments are half-staggered, based on data recently reported by Cohen et al , (1971) from X-ray diffraction studies of the A tubule of sea urchin sperm flagella The subunits proposed to contain either tubulins 1 or 2 are represented by white or black balls, respectively. Each protofilament is composed entirely of one type of tubulin, and protofilaments containing the same type of tubulin are grouped together in pairs, except in the partition region, where *three* protofilaments containing tubulin 1 are grouped together Other evidence which suggests that each nonpartition protofilament is composed of one type of tubulin comes from studies in which very small percentages of the outer portion of the B tubule have been removed and the solubilized protein subjected to quantitative acrylamide gel electrophoresis For example, if only 3–4% of the outer portion of the B tubule is removed (an amount equivalent to less than one protofilament), only tubulin 1 is observed on acrylamide gels

Although this model was constructed solely on the basis of the quantitative electrophoretic and electron microscope analyses described above, it is in good agreement with independent ultrastructural observations on the association of the protofilaments of the A tubule As noted in the preceding report partially-solubilized A tubules were observed to separate into one group of three proto-

[2] Recently, Warner (unpublished results) has corroborated Ringo's analysis by observations on negatively-stained outer doublets of clam gill cilia and has demonstrated 13 protofilaments in the A tubule and 10 in the B tubule.

filaments and several groups of two protofilaments (see Fig 16, Witman et al., 1972). These groups coincide with the group of three protofilaments in the partition region which contain only tubulin 1 and with the pairs of protofilaments in the remainder of the A tubule which are proposed to contain alternately tubulin 1 or 2. This correspondence between the biochemically distinct groups of protofilaments in the model and the groups actually observed with the electron microscope provides indirect support for the model. Furthermore, this correspondence permits tentative conclusions to be drawn with regard to the relative strengths of bonds between the same and different types of tubulins. The observation that the groups of two and three protofilaments detached from each other without separating into their individual protofilaments during breakdown of the A tubule indicated that the lateral bonds connecting individual protofilaments *within* a group were "stronger" than the lateral bonds *between* groups of protofilaments. If the model is correct that protofilaments within a group contain the same tubulin while protofilaments of alternate groups contain different tubulins, then these "strong" and "weak" bonds would correspond to bonds between like and unlike subunits, respectively.

Although outer doublet microtubules have a unique morphology because of the partition or shared middle wall, the more general features of a model for outer doublets should also be applicable to single microtubules Thus, if the arrangement diagrammed in Fig. 9 (A) is correct for outer doublets, one might expect that in a cytoplasmic microtubule having 12 protofilaments,[3] alternate pairs of protofilaments would contain the same type of tubulin (see Fig. 11), similar to the proposed arrangement for the nonpartition regions of the outer doublet Such an arrangement would give the microtubule a 3-fold radial symmetry and, as illustrated in Fig. 11, might account for the positioning of the links between the hexagonally arranged microtubules of *Brooklynella* (Lom and Corliss, 1971), *Nassula* (Tucker, 1968), *Saccinobaculus* (Grimstone and Cleveland, 1965) and other organisms [4]

[3] Roth (personal communication) has recently obtained definitive evidence that, in at least the heliozoan *Echinosphaerium*, single microtubules are composed of 12 protofilaments.

[4] We thank Dr. Richard McIntosh for first suggesting this possibility.

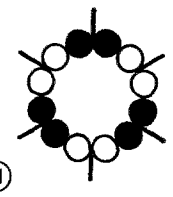

FIGURE 11 Diagram illustrating how the two tubulins might be arranged in a single microtubule having 12 protofilaments. Such an arrangement would give the microtubule a 3-fold radial symmetry and, as indicated, might account for the positioning of the six intermicrotubular links observed in some organisms.

On the other hand, there is evidence that some single microtubules contain 13 rather than 12 protofilaments. The central tubules of most cilia and flagella appear to be among these (Ringo, 1967). In addition, Warner (unpublished results) has recently demonstrated the presence of 13 protofilaments in the central tubules of gill cilia of the clam *Eliptio* by counting them in negatively-stained preparations. The "homofilament" model presented for the outer doublets in this paper (each protofilament containing a single tubulin, Figs. 8, 10, 11) can only be constructed when there are an even number of protofilaments. It there are an odd number of protofilaments (i e. 13) the only type of tubule which can be constructed is one composed of "heterofilaments" (each protofilament being composed alternately of subunits containing tubulin 1 and tubulin 2) as has been discussed by Bryan and Wilson (1971). It would seem that the number of protofilaments composing microtubules can vary with a resulting variation in the arrangement of the tubulins in the protofilaments. Thus, in *Chlamydomonas* axonemes, the outer doublet microtubules may be composed of homofilaments (if the three similar partition protofilaments are excluded, there remain 20 protofilaments in the outer doublet, 10 in the A tubule, and 10 in the B tubule) while the central microtubules may be composed of heterofilaments

Microheterogeneity within Tubulins 1 and 2

Microtubule protein isolated from several different sources by a variety of techniques occurs as a 110,000 dalton tubulin dimer (cf Renaud et al, 1968; Shelanski and Taylor, 1968; Weisenberg et al., 1968). This dimer appears to be a necessary intermediate unit in the assembly of protofilaments of microtubules in vitro (Borisy, 1970). As noted above,

when outer doublets are treated with detergent, the A tubule breaks down into one group of three protofilaments and several groups of two protofilaments Each group, if the model is correct, contains only a single type of tubulin; consequently, each dimer might be considered to contain only a single type of tubulin.[5] The outer doublet would then contain two types of homodimers—one composed of tubulin 1 subunits and one composed of tubulin 2 subunits. A heterodimer composed of one subunit each of tubulin 1 and 2 would appear to be excluded, particularly for the partition region, where three adjacent protofilaments contain only tubulin 1.

Nevertheless, certain biochemical characteristics of native microtubule protein, such as the presence of 1 mole of exchangeable and 1 mole of tightly bound guanine nucleotide per mole of dimer (Weisenberg et al , 1968), could be more easily explained if the dimer consisted of two different polypeptide chains If the dimer does indeed contain two different protein subunits, then each group of protofilaments which apparently contains only tubulin 1 may actually contain two different proteins which coelectrophorese on urea or SDS-urea gels as tubulin 1, and likewise each group which contains only tubulin 2 may actually contain two proteins which migrate together as tubulin 2. The entire outer doublet would then contain at least four different proteins.

To investigate this possibility, outer doublet proteins were analyzed by isoelectric focusing in polyacrylamide gels containing 8 M urea (Dale and Latner, 1968; Fawcett, 1960; Wrigley, 1968). After reduction and alkylation, outer doublet proteins could be focused into five major bands (Fig. 12) Physical and chemical characterization (peptide analysis, amino acid sequencing, etc.) of the protein in each band will be necessary to determine if these bands actually represent discrete polypeptide chains, or if the observed heterogeneity is caused by differences in carbohydrate or nucleotides which may be attached to one or both of the electrophoretically separable tubulins 1 and 2. Studies to be reported in detail elsewhere (Carlson, Witman, and Rosenbaum, in preparation) indicate that electrophoretically pure tubulin 1 and tubulin 2 each contain two or three bands on isoelectric focusing. Furthermore, certain bands were found in higher concentrations when different fractions of the outer doublet were analyzed: for example, only one band was observed in preparations of partition protofilaments (Fig 13, *P*), and when the central tubules and outer portion of the B tubules were removed and analyzed, two of the five bands became more prominent (13, *S*). If further studies should demonstrate that outer doublets actually contain five different proteins, then this result would suggest that only one of these proteins makes up the unique partition protofilaments while the remaining four proteins form two different types of heterodimers which are contained in the remainder of the A and B tubules

The general features of such an outer doublet construction should also extend to other types of microtubules. Thus, single microtubules (such as neurotubules or the central microtubules of the flagellum) should contain *four* different proteins which form two types of heterodimers Recent studies on tubulins from immature mouse brain (Feit et al., 1971) and neuroblastoma cells (Olmsted et al., 1971; Carlson, Witman, and Rosenbaum, in preparation) have shown that electrophoretically pure tubulin from either source separated into four different bands when analyzed by isoelectric focusing This data, therefore, suggests that single microtubules as well as outer doublet microtubules, may be constructed of two different types of heterodimers

SUMMARY

The findings reported here indicate that (*a*) two tubulins are present in *both* the A and B tubule of the outer doublets of *Chlamydomonas*, (*b*) only one of these tubulins is contained in the three protofilaments which make up the partition or wall between the lumens of the A and B tubule; (*c*) in the remainder of the outer doublet, protofilaments containing the same tubulin may be grouped together in pairs, the pairs containing tubulin 1 alternating with the pairs containing tubulin 2 (see Fig. 9 [A]).

Preliminary data from isoelectric focusing studies suggests that tubulins 1 and 2 may represent two *classes* of tubulins, and that each class contains two to three different tubulins These tubulins might interact to form at least two different types of heterodimers.

[5] The alternative is that one subunit of a dimer is located in one group of protofilaments and the other subunit in the adjacent group, and the dimer is split apart when the groups separate.

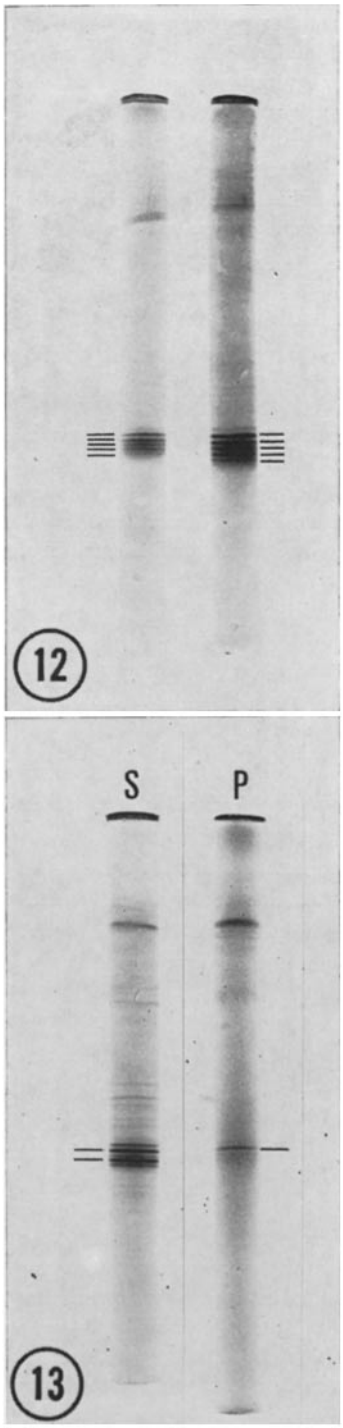

FIGURE 12 Isoelectric focusing of reduced and alkylated tubulins from intact outer doublets in polyacrylamide gels containing 8 M urea. Five major bands (bars) were consistently observed when varying amounts of protein were applied to the gels. The dark band in the upper half of the gels was not present in gels of electrophoretically pure tubulins. Left gel, 20 μg outer doublet protein; right, 40 μg. Ampholine range pH 4–6; Coomassie blue stain.

FIGURE 13 Isoelectric focusing of the proteins of isolated partition protofilaments (*P*). Only one major band (bar) was observed. An isoelectric gel (*S*) of a TEM matrix fraction (see preceding report by Witman et al., 1972) is included to allow comparison of the relative location of the partition band and of the other microtubule bands and to show that when only the central tubules and a portion of the B tubule are solubilized certain of the bands (bars) are more prominent than others. Conditions same as in Fig. 12.

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APPENDIX

NOMENCLATURE OF THE TUBULINS

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The two tubulins of the outer doublet microtubules of flagella were originally called tubulins *A* and *B* because it was thought that one composed the A tubule and the other the B tubule (Stephens, 1970) This nomenclature had to be discarded when it was found that the A tubule and the B tubule each contained both tubulins (Witman, 1970) It was suggested, therefore, that the tubulins be renamed tubulins 1 and 2 (Olmsted, et al, 1971). At the time this suggestion was made, workers began to report that microtubules from other sources e g neurons (Feit et al., 1971; Olmsted et al, 1971, Bryan and Wilson, 1971) and the mitotic apparatus (Bibring and Baxandall, 1971) also contained two tubulins and terminologies such as α, γ, and *alpha, beta* were used to denote the tubulins Since the two tubulins may represent classes of tubulins (Witman et al., this report) we propose that the major classes continue to be called tubulins 1 and 2 and that subclasses be called 1 α,β, and 2 α,β

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