# IDENTIFICATION OF MICROTUBULE-ASSOCIATED PROTEINS IN THE MEIOTIC SPINDLE OF SURF CLAM OOCYTES

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ABSTRACT

Meiotic spindles isolated from surf clam oocytes to morphological purity are biochemically complex, consisting of many polypeptides. These proteins fall into two classes: (*a*) polypeptides that are apparently cytoplasmic proteins and are not specifically associated with the spindle; and (*b*) polypeptides that are specifically associated with the spindle. A subset of the spindle-associated proteins, including a 250,000 mol wt component, remain with spindle tubulin through cycles of cold depolymerization and warm polymerization, showing that they are microtubule-associated proteins.

KEY WORDS mitosis · meiotic spindle · microtubules · microtubule-associated proteins · surf clam oocytes

Microtubules are the principal structural elements in the mitotic spindle and may themselves be the site of force generation for chromosome movement. For reviews see Goldman et al. (8) and Inoué and Stephens (10). Just how the microtubules form spindle fibers and associate with chromosomes or possibly control their movements is not yet understood, but some of these processes appear to depend on microtubule interactions which may in turn be determined by intertubule cross bridges. The goal of this paper is to biochemically identify the spindle proteins by isolating spindles, purifying spindle microtubules, and determining their composition by SDS polyacrylamide gel electrophoresis.

Three new observations are reported which help define the complexity of spindle composition and which will be of use in isolating spindle microtubules and in distinguishing microtubule cross bridges: (*a*) Meiotic spindles of surf clam oocytes can be isolated that are stable at 22°C but labile at 0°C and that can therefore be used for studies on spindle microtubule assembly in vitro. (*b*) Isolated spindles are complex biochemically. Many proteins are common to both the cytoplasm and the spindle but only a portion of them may actually be specifically associated with the spindle. (*c*) Some of the proteins that show an affinity for spindles, including one electrophoretic component of 250,000 mol wt, display the properties of microtubule-associated proteins (MAPs).[1]

## MATERIALS AND METHODS

### *Isolation of Meiotic Spindles*

Ovaries of the surf clam, *Spisula solidissima* (obtained from the Supply Department, Marine Biological Laboratory), were

[1] *Abbreviations used in this paper:* MAP, microtubule-associated protein; MES, 2-(*N*-morpholino)ethane sulfonic acid; PIPES, piperazine-*N,N'*-bis(2-ethane sulfonic acid).

teased apart and the oocytes were filtered through gauze, washed several times in filtered seawater, and stored in large finger bowls until use. The oocytes were activated at 18°–20°C by increasing the potassium ion concentration of the seawater by 38 mM by the addition of an isotonic stock solution containing 0.52 M KCl in distilled water. As described by Allen (1), this treatment induces the oocytes to undergo the first and second meiotic divisions after 25–30 min at 18°C. To obtain complete and synchronous activation of the oocytes it was essential to use only ripe animals, to rinse the oocytes in filtered seawater at least three times immediately before activation, and to maintain conditions of low cell density (1 ml packed oocytes/20 ml seawater). If these precautions are not taken, the spindles are contaminated with membranes, pieces of cell cortex, and germinal vesicles. At 16 min after activation (or when all oocytes exhibited a large meiotic metaphase spindle), the following steps were performed in rapid succession: (*a*) Oocytes were pelleted (1 min, 1,500 rpm) in a benchtop centrifuge and resuspended in 20 volumes of 30°C medium containing 1 mM potassium phosphate, pH 8.0, and 1 M glycerol to remove vitelline membranes and to disperse hyaline (19, 21). (*b*) Cells were centrifuged (1 min, 500 rpm) to bring most of the cells to the bottom third of the tube, the supernate was removed by aspiration, and the loose cell pellet was resuspended in 3 vol of 30°C lysis medium based on that of Sakai et al. (22) containing glycerol and Triton X-100. The isolation medium contained 5 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), pH 6.2, 5 mM EGTA, 0.5% Triton X-100, and 1 M glycerol. (*c*) The suspension was immediately and vigorously shaken in a large flask to lyse the oocytes and disperse the cytoplasm. With practice, it was possible to perform these steps within 7 min. Meiotic spindles were immediately isolated by centrifugation (5 min, 3000 $g_{(max)}$, 23°C).

## *Preparation of Spindle Extract and Isolation of Microtubules by In Vitro Polymerization*

The spindle pellet was resuspended in an equal volume of double-strength microtubule assembly buffer to provide the following concentrations of factors after resuspension: 0.1 M piperazine-*N*,*N*′-bis(2-ethane sulfonic acid) (PIPES), pH 6.94, 0.1 mM $Mg^{++}$, and 1.0 mM GTP. The suspension was brought to 0°C for 30 min to depolymerize microtubules and centrifuged (20 min, 37,000 $g_{(max)}$, 5°C) to produce a cold soluble spindle extract containing tubulin. Microtubules were assembled at 30°C and purified by two cycles of the in vitro assembly procedure described by Borisy et al. (2) for the isolation of microtubule protein[2] from porcine brain tissue.

## *SDS Gel Electrophoresis*

Acrylamide gels (5%, 0.5 × 8.0 cm) were prepared according to the methods of Shapiro et al. (25) for continuous gels containing 0.1 M phosphate, pH 7.2, and of Bryan (3) for gels containing 25 mM Tris-glycine, pH 8.3. Gels were stained for protein with Coomassie blue R250 or for carbohydrate by the periodic acid-Schiff (PAS) reaction according to the procedures of Fairbanks et al. (5) and scanned at 560 nm with a Gilford spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). Areas under the peaks were determined by cutting and weighing the peak areas from photocopies of the original gel tracings. For Coomassie blue staining, peak area was previously determined to be proportional to the tubulin concentration over a wide range of concentrations (2).

## *Electron Microscopy*

For negative staining, samples (5 μl) were placed on 400-mesh grids coated with Formvar and carbon and negatively stained by successively displacing the sample with four drops each of the following solutions: 1 mg/ml cytochrome *c* in distilled water, distilled water, 1% aqueous uranyl acetate. Excess stain was removed with a filter paper.

For analysis of thin sections of embedded pellets of microtubules by electron microscopy, 2 mg of polymerized protein was centrifuged (225,000 $g_{(max)}$ at 25°C for 30 min) in 8-ml polycarbonate Oak Ridge tubes, fixed in 2.5% glutaraldehyde in assembly buffer, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in acetone, and embedded in Araldite as described previously (17). Sections were stained in methanolic uranyl acetate and in lead citrate and examined at 60 kV on a Philips 201 electron microscope.

## *Other Biochemical Procedures*

Microtubule protein was isolated from beef brain by the method of Borisy et al. (2) as modified by Murphy and Hiebsch (18) in 0.1 m PIPES, pH 6.62, containing 1 mM EGTA, 0.1 mM $Mg^{++}$, and 1.0 mM GTP. *Spisula* sperm tail axonemes were prepared by an adaptation of the method of Gibbons (7) and of Stephens (26) for isolating sea urchin sperm flagellar axonemes. Protein concentration was determined by the method of Lowry et al. (15) using bovine serum albumin as a standard.

# RESULTS

## *Conditions Required for Spindle Isolation*

To purify spindle microtubules by in vitro assembly, it was first necessary to obtain preparations of spindles that were labile at 0°C but stable enough at 22°C that they could be isolated by differential centrifugation. Because isolation media containing solvents such as hexylene glycol (11) or dimethyl sulfoxide (6) produced cold-stable microtubules, spindles were isolated by a modification of the method of Sakai (22) which yields spindles that are cold-labile. EGTA, $Mg^{++}$, glycerol and low pH were all found to increase spindle stability. However, EGTA and $Mg^{++}$ were used at low concentrations, since in *Spisula* 20 mM EGTA and 0.1 mM $Mg^{++}$ were found to induce clumping of the cytoplasm and to stabilize membranes and the cell cortex. Glycerol, on the other hand, stabilized spindles but did not induce cytoplasmic clumping. Therefore, the isolation medium that was employed contained 5 mM MES, pH 6.2, 5 mM EGTA, 0.5% Triton X-100, 1 M glycerol, and no $Mg^{++}$.

[2] In this paper, microtubule protein refers to all of the microtubule components obtained by the in vitro assembly procedure including both tubulin and nontubulin proteins.

The morphology of the isolated spindles is shown in Fig. 1 *a* and *b*. Isolated spindles looked like spindles in oocytes before lysis. The poles were well defined and spindle fibers were distinct, with each phase-dense fiber corresponding to a birefringent fiber as seen by polarization microscopy. As the individual spindle fibers and their arrangement in the spindle were well preserved, it seemed likely that the molecular components responsible for maintaining spindle structure were present in the isolated structures.

An example of a spindle pellet as viewed by both phase-contrast and polarization microscopy is shown in Fig. 2 *a* and *b*. In most cases, each phase-dense object corresponded to a birefringent spindle as seen by polarization microscopy. All preparations were contaminated somewhat with membranes, cell cortex, and hyaline which could not be removed by washing but the contamination was minimal when the precautions outlined in Materials and Methods were taken for activating and lysing the oocytes. As described below, washing did remove a few of the particular contaminants but the majority of the protein (85–90%) remained with the spindles.

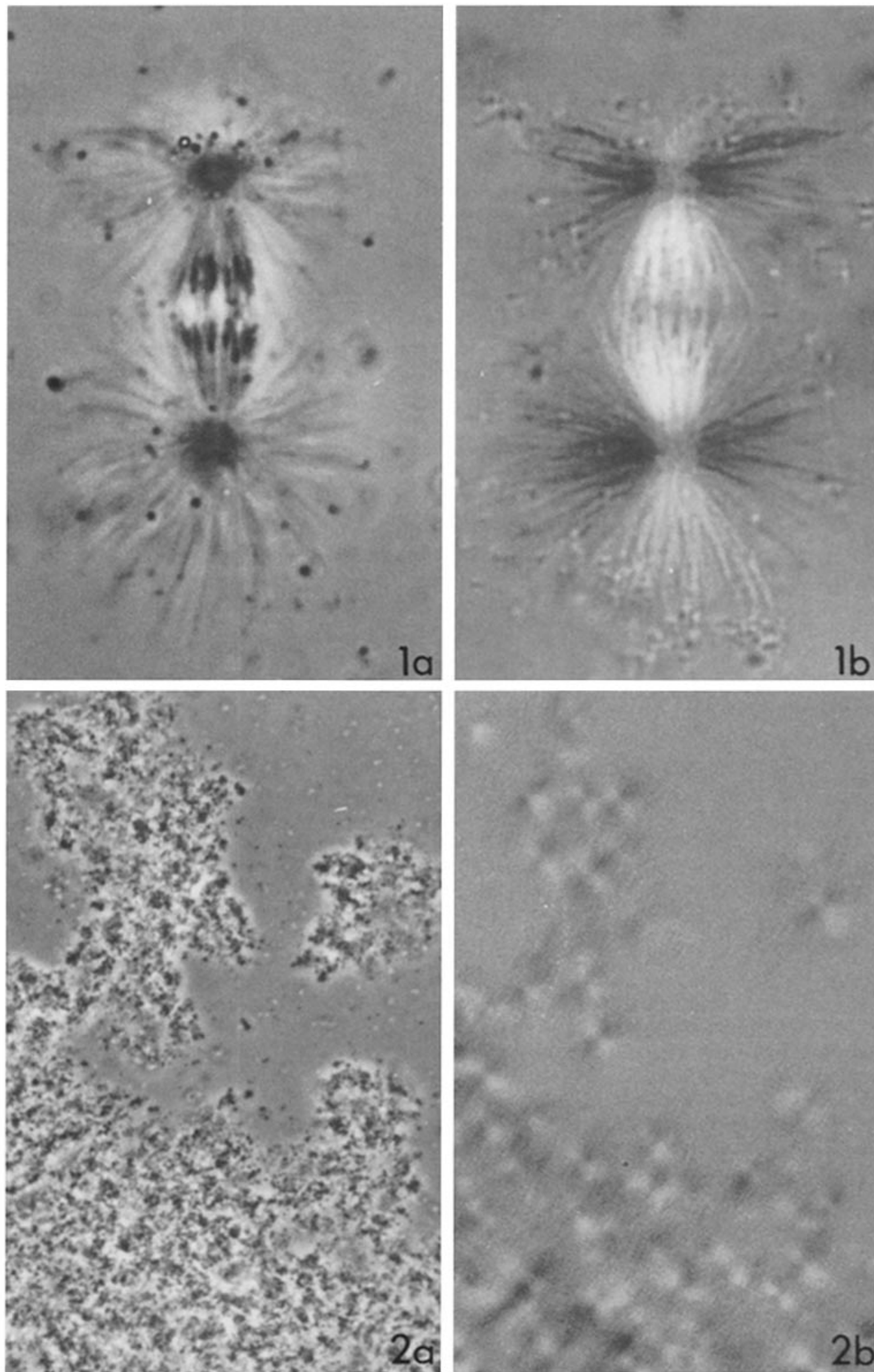
### *Determination of Spindle Composition*

SDS gels of isolated spindles contained dozens of different electrophoretic components which for reference have been assigned numbers beginning with those components of highest molecular weight (Fig. 3 *a*). The electrophoretic mobility of P1 was indistinguishable from that of axonemal dynein (350,000 mol wt); P3 and 4 (250,000 mol wt) were similar to the high molecular weight MAPs from brain, and P12 was similar to actin (43,000 mol wt). The 55,000 mol wt band identified as P11 was concluded to be tubulin because (*a*) it comigrated with tubulin in axonemes and neuronal microtubules; (*b*) it split into separate $\alpha$ and $\beta$ components on Tris-glycine gels; (*c*) the band was depleted when spindles were extracted under conditions that depolymerized spindle microtubules; (*d*) as described subsequently, the microtubules which polymerized when the spindle extract was incubated under conditions that favored microtubule assembly were enriched in this polypeptide. In general, the same electrophoretic components that were the primary factors in the spindle (Fig. 4 *b*) were also primary components in the whole cell lysate (Fig. 4 *a*). To determine which proteins were nonspecific cytoplasmic contaminants and which ones were really spindle-associated, spindles were examined in the following three ways.

COMPARATIVE GEL ANALYSIS OF SPINDLES ISOLATED IN GLYCEROL AND IN HEXYLENE GLYCOL: Spindles isolated in glycerol were compared to spindles isolated in hexylene glycol to determine which polypeptides were specific spindle components. The rationale for this experiment is as follows: Hexylene glycol spindles are cleaner than glycerol spindles when compared either by microscopy or by electrophoresis. However, if spindle proteins are retained in both preparations (both preparations do contain distinct spindle fibers), then the mass ratio of the spindle proteins to tubulin would be expected to be the same for both preparations regardless of other differences in composition caused by cytoplasmic contamination. Thus, for the spindle-specific proteins, the ratio of the mass ratios from both preparations would be 1, whereas for nonspecific proteins the ratio would be greater or less than 1.

From visual comparison of SDS gels both spindle preparations were found to have the same electrophoretic components (Fig. 3 *a* and *b*). However, in examining the ratios of the mass ratios of each of 15 nontubulin components relative to tubulin, it was found that some components including P1, 3, 4, 10, and 15 had ratios ranging from 1 to 2, suggesting that these proteins may be specific spindle proteins (Table I). However, proteins P2, 5, 6, 7, 9, 12, 14, and 16 had ratios >2, indicating that these proteins may be nonspecific cytoplasmic components.

WASHING SPINDLES BY RESUSPENSION TO REMOVE ADHERENT PARTICLES: When preparations of stabilized glycerol spindles were resuspended in lysis medium and then allowed to settle, the supernatant wash was observed to contain many cytoplasmic granules but no spindles. SDS gels of washed and unwashed spindles were identical. This is not surprising as washing removed only 5–10% of the protein (as determined by Lowry assay) and because the washed spindles contained adhering particles. However, the supernate from the second consecutive wash appeared to be greatly enriched in proteins P6, 7, and 9 (Fig. 4 *c*). This was substantiated by comparing the mass ratios of the nontubulin proteins to tubulin in the supernatant wash and in the unwashed spindles by comparative electrophoresis. For most proteins, the ratio of the mass ratios was ~1, indicating that



1a
1b
2a
2b

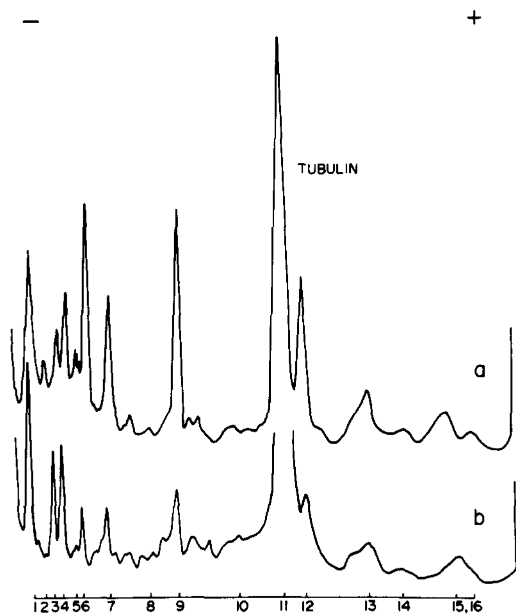



FIGURE 3 Electrophoretic analysis on 5% acrylamide gels of spindles prepared in medium containing glycerol (*a*) and hexylene glycol (*b*). Gels from two preparations of hexylene glycol spindles and from six preparations of glycerol spindles (all prepared from different animals) looked similar. Both types of preparation also varied with respect to the amount of hyaline they contained. Tubulin comprised 30 and 45% of the glycerol and hexylene glycol preparations, respectively.

these proteins were about as common in the supernatant wash as they were in the spindles. However, for P6, 7, and 9 as well as for P10 and 13–16, ratios >2 were observed, suggesting that these proteins may correspond to the particulates observed by microscopy.

PAS STAINING OF SPINDLE EXTRACTS: Spindle proteins P6, 9, and 12 gave strong PAS-positive reactions, and proteins P7, 10, and 13 gave moderate to weak reactions in the three spindle extracts that were examined, suggesting that at least three major components of the spindle (P6, 9, and 12) may be nonspecific cytoplasmic proteins (Fig. 5).

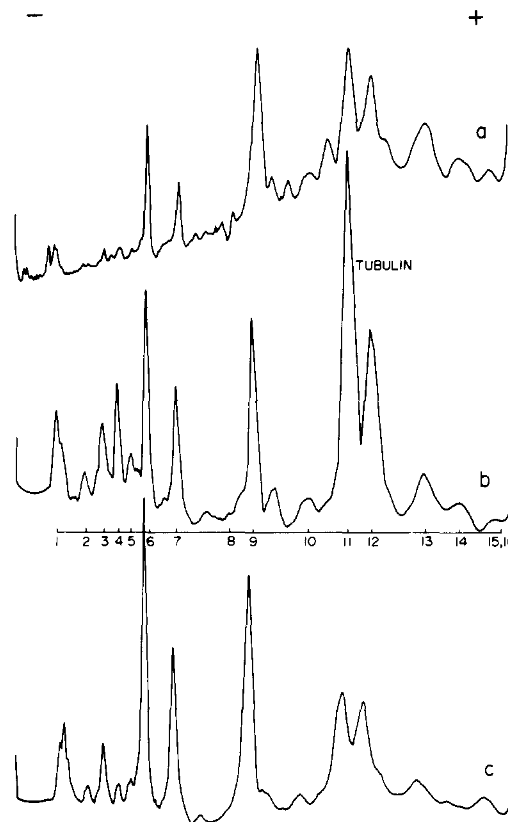



FIGURE 4 Electrophoretic analysis of spindles isolated in 1 M glycerol. Densitometer tracings of 4% polyacrylamide gels containing (*a*) whole cell lysate, (*b*) isolated spindles, and (*c*) particle-rich supernate obtained from resuspended spindles after the second consecutive resuspension in lysis medium. The primary electrophoretic components identified in the preparation of isolated spindles in *b* are indicated by number, beginning with the components of highest molecular weight. Protein P11 is tubulin.

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FIGURE 1 Stabilized meiotic spindle in cell lysate. (*a*) Phase-contrast and (*b*) polarization microscopy. Immediately after lysis, spindles were further stabilized by adjusting the lysate to 2.0 mM $mg^{++}$ and 3 M glycerol. In the absence of magnesium, spindle fibers remained intact, but the chromosomes dispersed and disappeared within a few minutes. The isolated spindles are free of most adherent material and have well-defined astral and chromosomal fibers. × 2,500.

FIGURE 2 Resuspended spindle pellet. (*a*) Phase-contrast and (*b*) polarization microscopy. After cell lysis, the stabilized spindles were centrifuged and resuspended in isolation medium containing 3 M glycerol and 2 mM $Mg^{++}$ at 22°C. In most cases, each phase-dense object corresponded to a birefringent spindle component as seen by polarization microscopy. × 540.

Table I

*Comparison of the Mass Ratio of Spindle Proteins to Tubulin in Preparations of Spindles Isolated in Glycerol and in Hexylene Glycol*

| Protein | A Glycerol procedure | | B Hexylene glycol procedure | | A/B |
|---|---|---|---|---|---|
| | Peak area (arbitrary units) | Ratio to tubulin | Peak area (arbitrary units) | Ratio to tubulin | |
| 1 | 200 | 0.256 | 240 | 0.199 | 1.3 |
| 2 | 51 | 0.065 | 5 | 0.004 | 16.2 |
| 3 | 92 | 0.118 | 75 | 0.062 | 1.9 |
| 4 | 124 | 0.159 | 111 | 0.092 | 1.5 |
| 5 | 88 | 0.113 | 50 | 0.041 | 2.7 |
| 6 | 206 | 0.264 | 12 | 0.010 | 26.0 |
| 7 | 164 | 0.210 | 47 | 0.039 | 5.4 |
| 8 | 24 | 0.030 | 30 | 0.024 | 1.3 |
| 9 | 243 | 0.312 | 120 | 0.100 | 3.1 |
| 10 | 69 | 0.088 | 103 | 0.085 | 1.0 |
| 11 Tubulin | 780 | 1.000 | 1205 | 1.000 | 1.0 |
| 12 | 246 | 0.315 | 175 | 0.145 | 2.2 |
| 13 | 162 | 0.208 | 133 | 0.110 | 1.9 |
| 14 | 103 | 0.132 | 27 | 0.022 | 6.0 |
| 15 | 104 | 0.130 | 90 | 0.072 | 1.8 |
| 16 | 35 | 0.044 | 18 | 0.014 | 3.1 |

## *Microtubule Self-Assembly in Spindle Extracts*

Spindle extract was prepared from cold depolymerized spindles by high-speed centrifugation (30 min, 37,000 $g_{(max)}$ at 5°C). Because the isolated spindles dissociated within 30 min at 22°C and because the maximum possible tubulin concentration was desired, unwashed spindles were used which contained 10% tubulin. The spindle extract (Fig. 5 *a*) prepared from these spindles was free of hyaline and particulate material and contained 15–25% tubulin and in general was similar in composition to intact washed spindles (Fig. 3 *a*).

When spindle extract in 0.1 M PIPES at pH 6.94 was warmed to 30°C for 20 min, microtubules self-assembled as determined by negative staining and electron microscopy (Fig. 6). In addition to microtubules, flocculent material and some beaded aggregates were also observed. These microtubules were pelleted, resuspended in assembly buffer at 0°C, and carried through one additional cycle of disassembly and assembly to obtain a second pellet of assembled microtubules. When material from the second cycle of polymerization was examined by electron microscopy after negative staining, several polymer forms in addition to microtubules were observed. When this polymer was pelleted, fixed, and sectioned for microscopy (Fig. 7), microtubules and structures such as partially closed microtubules, sheets and beaded aggregates were observed. Thus, the efficiency of assembly in the second cycle as judged by the ability to form microtubules was reduced.

The yield of tubulin obtained from spindle extract after two cycles as determined by sedimentation and gel electrophoresis is shown in Table II. From ~7 ml of packed oocytes, an initial lysate was obtained containing 25.8 mg tubulin (5.2% of the total cell protein). Of this, 2.4 mg (9.3% of the tubulin) pelleted as isolated spindles, and 0.9 mg (3.5%) was obtained as cold-soluble spindle extract. The yield of tubulin, based on the amount of tubulin contained in the extract which was designated 100%, was 83 and 13% for the first and second microtubule pellets, respectively.

## *Identification of MAPs*

SDS gels of microtubule protein obtained by in vitro purification are shown in Fig. 8 *a–d*. To aid in identifying possible MAPs, the fractional ratios of each of the principal electrophoretic components to tubulin vs. the stage of purification were determined as shown in Fig. 9. In this experiment,

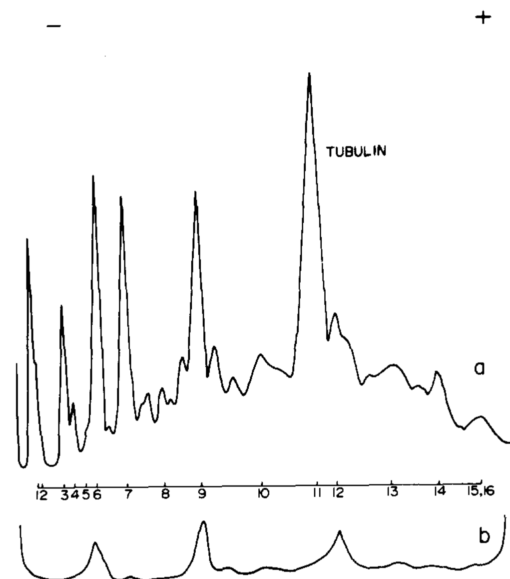


Figure 5 PAS staining of spindle extract. Densitometer tracings of gels containing spindle extract stained in (*a*) Coomassie brilliant blue or in (*b*) PAS. The numbers mark electrophoretic components that are indicated in the text.

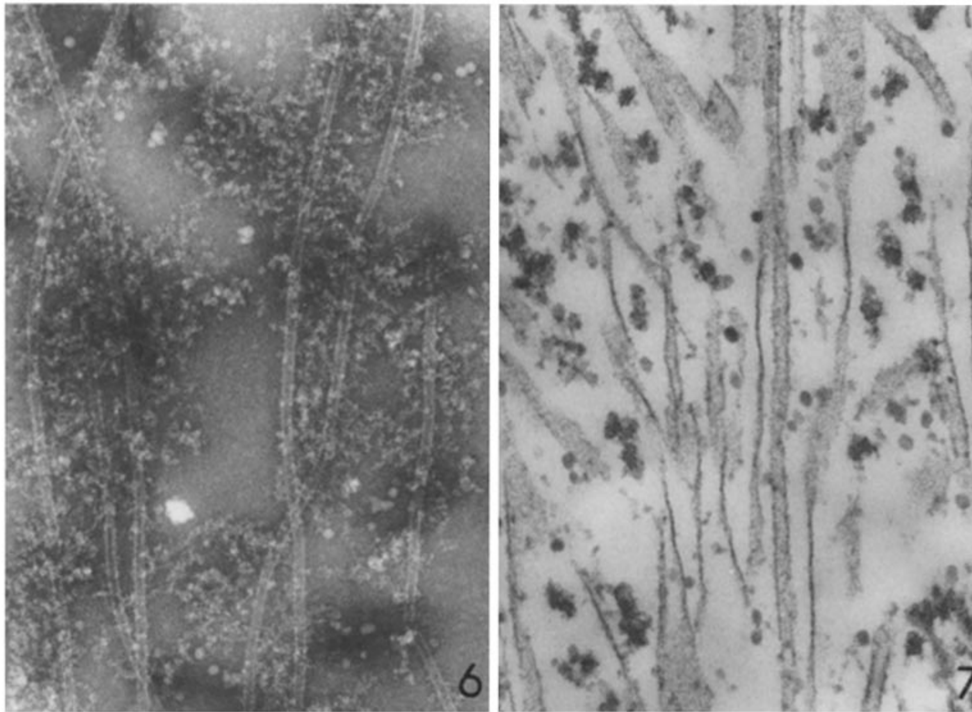

FIGURE 6 Negatively stained microtubules in spindle extract after in vitro polymerization at 30°C. × 46,200.

FIGURE 7 Microtubule polymer after second cycle of in vitro polymerization. Section of fixed embedded pellet. × 74,500.

which was typical of the experiments examined, tubulin was rapidly purified, increasing from 18.2% in the extract to 38.3% in the first tubule pellet, and 68.8% in the second tubule pellet (Table III). Most components, including many of the major electrophoretic components such as P1, 6, 7, and 9, were rapidly purified away. However, a few components including P3, 4, 8, and 13 were depleted less rapidly, thus demonstrating a higher affinity for tubulin.

## DISCUSSION

### *Purification of Spindle Microtubule Protein*

It was important to consider the concentration of glycerol employed in the spindle isolation medium, as this solvent favored the stabilization of spindles but depleted the amount of MAPs in polymer obtained by in vitro assembly. Previous studies on brain tubulin assembly demonstrated that the presence of 3.4 M glycerol in tubule assembly buffer reduced the amount of MAPs (24), and that tubulin purified by ion exchange chromatography will self-assemble in glycerol (14) and in other solvents (9) in the absence of any MAPs. In agreement with this finding is the recent report by Keller and Rebhun (13) in which it is stated that preparations of microtubules purified from isolated echinoderm spindles using assembly medium containing 3.4 M glycerol contained only trace amounts of MAPs. In the present paper, glycerol was used to isolate spindles that were cold-labile. It is possible to isolate spindles in medium lacking glycerol (20, 23), but in the case of *Spisula* it was found necessary to use other stabilizing agents such as $Mg^{++}$ or EGTA both of which resulted in membrane contamination that was greater than when glycerol alone was used. Using medium containing 1 M glycerol, it was possible to isolate spindles that were stable at 22°C for up to 30 min and also to obtain microtubules that contained MAPs after purification by in vitro assembly from spindle extracts.

TABLE II
*Purification of Tubulin from Spindle Extract**

| Sample‡ | Volume | Concn | Protein | Tubulin§ | Tubulin | Yield |
|---|---|---|---|---|---|---|
| | *ml* | *mg/ml* | *mg* | *%* | *mg* | *%* |
| Lysate | 145 | 3.4 | 495 | 5.2 | 25.80 | |
| Spindles | 1.4 | 18.8 | 26.3 | 9.1 | 2.40 | 100.0 |
| Spindle Extract | 1.2 | 4.4 | 5.3 | 18.2 | 0.90 | 37.5 |
| $H_1P$ | 0.9 | 2.1 | 1.9 | 38.3 | 0.72 | 30.0 |
| $C_1S$ | 0.8 | 0.63 | 0.5 | 61.3 | 0.31 | 12.9 |
| $H_2P$ | 0.4 | 0.43 | 0.17 | 68.8 | 0.12 | 4.8 |

* The data are for a single isolation using 7 ml of packed oocytes.

‡ HS, HP, CS, and CP refer to the "hot" and "cold" supernate and pellets obtained by the in vitro assembly procedure for purifying microtubule protein. The numerical subscript refers to the number of cycles of purification.

§ The figures for percent tubulin represent the means of five different determinations.

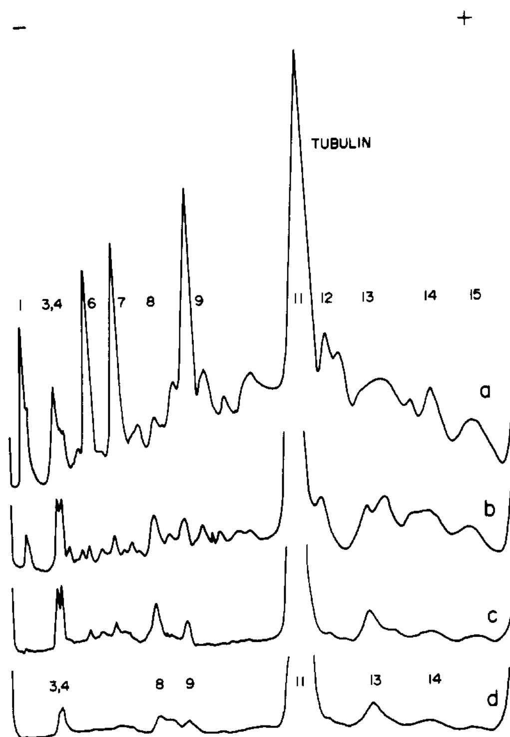


FIGURE 8 Compositional analysis of spindle microtubule protein purified by cycles of in vitro assembly and disassembly. (*a*) Cold-soluble spindle extract; (*b*) first microtubule pellet, $H_1P$; (*c*) supernate of cold-dissociated microtubule pellet, $C_1S$; (*d*) second microtubule pellet, $H_2P$.

### *On the Low Yield of Spindle Microtubule Polymer*

It was previously reported that microtubules do not polymerize in high-speed extracts of either sea urchin eggs (12) or surf clam oocytes (27) in glycerol-free microtubule assembly buffers used to purify microtubules from brain. One explanation provided by Bryan et al. (4) was that an inhibitory factor similar to RNA, which could be isolated from sea urchin eggs and which inhibited brain tubulin assembly, might normally inhibit microtubule self-assembly in non-neuronal cells. However, in the studies described here, extracts of isolated spindles were used which should have been free of most soluble RNA. Nevertheless, the

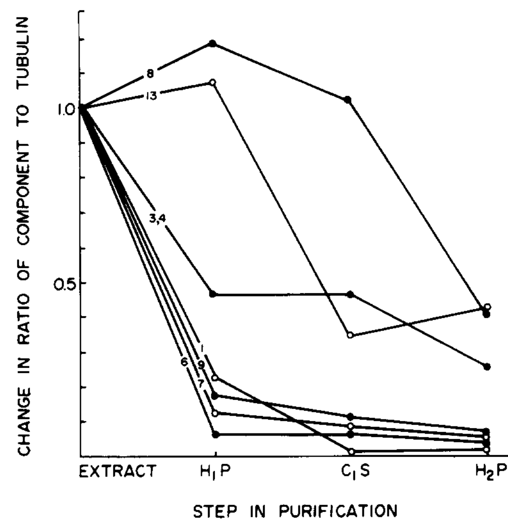


FIGURE 9 The co-purification of spindle proteins with tubulin. Microtubule protein was examined by quantitative gel electrophoresis to determine the stoichiometry of MAPs to tubulin through two cycles of in vitro assembly-disassembly. The amount of various electrophoretic components was determined from densitometer tracings of gels taken from each stage of purification. The ratio of each component to tubulin was determined, and is expressed at each stage as the percent of the ratio observed in the extract which was designated 100%.

TABLE III

*Comparison of the Mass Ratio of Spindle Proteins to Tubulin in a Preparation of Spindles and in a Supernate of Washed Spindles*

| Protein | A Supernatant wash | | B Spindles | | A/B |
|---|---|---|---|---|---|
| | Peak area (arbitrary units) | Ratio to tubulin | Peak area (arbitrary units) | Ratio to tubulin | |
| 1 | 183 | 0.464 | 237 | 0.262 | 1.8 |
| 2 | 52 | 0.132 | 94 | 0.104 | 1.3 |
| 3 | 133 | 0.338 | 196 | 0.217 | 1.6 |
| 4 | 57 | 0.145 | 184 | 0.203 | 0.7 |
| 5 | 59 | 0.150 | 136 | 0.150 | 1.0 |
| 6 | 357 | 0.906 | 266 | 0.294 | 3.1 |
| 7 | 251 | 0.637 | 205 | 0.227 | 2.8 |
| 8 | 25 | 0.063 | 52 | 0.058 | 1.1 |
| 9 | 468 | 1.188 | 326 | 0.361 | 3.3 |
| 10 | 84 | 0.213 | 75 | 0.083 | 2.6 |
| 11 Tubulin | 394 | 1.000 | 904 | 1.000 | 1.0 |
| 12 | 336 | 0.852 | 437 | 0.483 | 1.8 |
| 13 | 156 | 0.396 | 172 | 0.190 | 2.1 |
| 14 | 77 | 0.195 | 64 | 0.071 | 2.7 |
| 15, 16 | 129 | 0.327 | 24 | 0.027 | 12.1 |

yield of microtubules from spindle extracts was low compared with that of neuronal microtubules from brain tissue. For neuronal microtubules, it has been demonstrated that the yield of tubulin from brain extract was 16% after two cycles of in vitro purification in 0.1 M PIPES buffer at pH 6.94 (2). However, with the same assembly buffer, the yield of tubulin from the spindle extract after two cycles of polymerization was only 4.8%. In addition, aberrant polymer forms (partially closed microtubules and sheets) were observed after two cycles of assembly of the spindle microtubule protein. It has been reported that the polymerization of neuronal microtubule protein at concentrations of protein close to the critical concentration required for polymerization results in the formation of various polymers, including sheets, C-shaped and S-shaped forms, and tubulin with partially closed walls (16). This could account for the aberrant polymer forms observed here because the tubulin concentrations in the spindle extract and the $C_1S$ fraction were estimated to be 0.8 and 0.4 mg/ml, respectively.

There are several possibilities for the reduced level of polymerization of the spindle protein. One possibility is that the tubules did not polymerize well in the extract. Although the first polymerization was good (80% of the extract tubulin was recovered in the $H_1P$ fraction), the second polymerization was not (37% of the tubulin in the $C_1S$ fraction was recovered in the $H_2P$ fraction). Reasons for this may be differences in the ionic requirements for spindle tubulin assembly, or a lower concentration of MAPs, or MAPs with a lower affinity for tubulin. None of these hypotheses has yet been examined.

Another possibility for the low yield of microtubules is that the tubules did not depolymerize in the cold. Only 37.5% of the tubulin present in the spindles was obtained in the extract, and 43% of the tubulin present in the $H_1P$ fraction was recovered in the $C_1S$ fraction. Thus, it is possible that cold-stable tubules formed that were resistant to low temperature or that some of the tubulin polymer assembled into aggregates and polymer forms that were not solubilized by low temperature. The first possibility seems unlikely, as no microtubules were observed when spindles treated with low temperature were negatively stained and examined by electron microscopy.

### *Identification of Proteins Specifically Associated with the Spindle*

Preparations of spindles were observed to contain dozens of proteins that could be distinguished on SDS gels. In general, the same electrophoretic components that were observed to be the major proteins in the spindle preparations were also observed to be primary constituents of the whole cell cytoplasm, and in fact it was possible to demonstrate this fact by comparative gel electrophoresis. As summarized in Table IV, of the 15 nontubulin components that were examined, three proteins were demonstrated to be probable cytoplasmic proteins, four were postulated to be MAPs, and eight proteins could not be assigned any group affiliation.

At least three proteins (P6, 7, and 9) appeared to be common to both the spindle and the cytoplasm and may be associated with the particulate material seen adhering to the spindles. This conclusion is supported by the observations that P6, 7, and 9 are reduced in amount in the cleaner hexylene glycol spindles, are present in the particle-rich supernate from washed spindles, and are strongly PAS-positive. It seems likely that these proteins may represent yolk or other membrane-containing particles that are nonspecifically adsorbed on the spindles. However, another possibility that cannot be excluded at this time is that

Table IV
*Affiliation of Proteins Present in Isolated Spindles*

| Exp | Protein | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Spindle comparisons | S | C | S | S | C | C | C | S | C | S | S | C | S | C | S | C |
| Resuspension-washing | S | S | S | S | S | C | C | S | C | C | C | S | C | C | C | C |
| PAS staining | | | | | | C | C | | C | C | | C | C | | | |
| In vitro polymerization | C | | S | S | | C | C | S | C | C | S | C | S | | | |

S, spindle affiliation; C, cytoplasmic affiliation.
Blanks indicate proteins whose affiliation could not be evaluated.

they are indeed important spindle-associated components, but it seems unlikely that they would be fibrous proteins directly related to spindle structure. One puzzling observation is that the extract also contained P6, 7, and 9. One fact that may account for this is the observation that a white layer of lipidlike material was always observed on top of high-speed extract, suggesting that particles containing yolk or lipoproteins may have been disrupted during the centrifugation.

Another set of proteins (P3, 4, 8, and 13) displayed some of the properties one would predict would be displayed by specific spindle-associated proteins: they partially copurified with tubulin during cycles of in vitro purification of spindle tubulin, they remained associated with the spindles during washing to remove adsorbed cytoplasmic proteins, they were solubilized when microtubules were depolymerized by exposure to low temperature, and they had similar ratios in preparations of spindles obtained using different isolation media. So far, it has not been possible to examine all of the trace species in as much detail, although clearly these components could also play important roles in determining spindle structure and function. At the present time, one should use some reservation in considering these proteins to be spindle MAPs, as it will be necessary to study their copurification behavior through several additional cycles of tubule assembly-disassembly and examine the ability of purified spindle MAPs to stimulate tubulin polymerization before the affinity of these proteins for tubulin is known in detail.

So far, none of the spindle microtubule proteins has been identified with other known contractile proteins or MAPs isolated from other cell types. Two of the high molecular weight spindle proteins have electrophoretic mobilities similar to those of other MAPs. One component, P1, of ~350,000 mol wt has an electrophoretic mobility indistinguishable from that of dynein from *Spisula* axonemes. Another spindle component (P3, 4) which is identified as a doublet band of 250,000 mol wt on SDS gels and copurifies with tubulin during in vitro assembly resembles the 270,000 mol wt doublet band (MAP-2) observed in gels of neuronal microtubules from brain tissue. In addition, there are other spindle components such as P8 (140,000 mol wt) and P13 (30,000 mol wt) that display some affinity for spindle tubulin but for which there are no known MAP correlates in brain. Clearly, further work on the composition, structure, and function of the spindle proteins is required to determine whether they are related to dynein or to MAPs and whether they determine the function of microtubules in mitosis.

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