SELECTIVE EXTRACTION OF ISOLATED MITOTIC APPARATUS

Evidence That Typical Microtubule Protein Is Extracted by Organic Mercurial

THOMAS BIBRING and JANE BAXANDALL

From the Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37203

ABSTRACT

Mitotic apparatus isolated from sea urchin eggs has been treated with meralluride sodium under conditions otherwise resembling those of its isolation. The treatment causes a selective morphological disappearance of microtubules while extracting a major protein fraction, probably consisting of two closely related proteins, which constitutes about 10% of mitotic apparatus protein. Extraction of other cell particulates under similar conditions yields much less of this protein. The extracted protein closely resembles outer doublet microtubule protein from sea urchin sperm tail in properties considered typical of microtubule proteins: precipitation by calcium ion and vinblastine, electrophoretic mobility in both acid and basic polyacrylamide gels, sedimentation coefficient, molecular weight, and, according to a preliminary determination, amino acid composition. An antiserum against a preparation of sperm tail outer doublet microtubules cross-reacts with the extract from mitotic apparatus. On the basis of these findings it appears that microtubule protein is selectively extracted from isolated mitotic apparatus by treatment with meralluride, and is a typical microtubule protein.

INTRODUCTION

The constituent proteins of microtubules from several cell types have recently been identified on the basis of their binding of colchicine (1–10), their precipitation by vinblastine (7–9, 11–13), and, in cilia and flagella, by selective solubilization procedures monitored by electron microscopy (3, 5, 14–19). These proteins show a striking resemblance in physicochemical properties (3–6, 9, 14–17) which serves to confirm the interpretation that they are in fact microtubule proteins.

Efforts to extract functionally significant proteins from isolated mitotic apparatus (20–29) commenced before the studies of microtubule proteins. The earliest studies of mitotic apparatus even preceded the identification of microtubules as the major morphologically recognizable element of spindle fibers. In extraction studies of mitotic apparatus, the general approach has been to disperse isolated mitotic apparatus entirely, most typically in approximately half molar potassium chloride. After potassium chloride treatment, only membranous vesicles can be recovered in particulate and morphologically unaltered form (24). Seemingly diverse proteins are found in the soluble extract, and have been partially or extensively characterized. Their similarity to microtubule proteins ranges from little similarity (24, 30, 31) to points of close similarity (17, 26, 32).

We have felt that the isolation and identification of microtubule protein from mitotic apparatus would be facilitated by extraction procedures intended to be specific for microtubule protein. At the ultrastructural level, such extractions should cause microtubules to disappear while leaving the rest of the mitotic apparatus intact (27, 33–35). We have described such an extraction procedure (27), but the soluble protein obtained proved difficult to characterize. It tended to aggregate rapidly, and its properties changed with minute changes in the medium, such as addition of 10^{-5} M calcium ion.

The procedure to be described here extracts protein in a more stable form. On the basis of the knowledge that organic mercurial depolymerizes outer doublet microtubules from sperm flagella (17, 27), and the hypothesis that microtubules of mitotic apparatus would react similarly, we added mercurial to isolated mitotic apparatus while otherwise keeping conditions close to those employed for isolation. Meralluride sodium, which is soluble at the slightly acid pH used, was the organic mercurial employed. As described below, this treatment causes a selective morphological disappearance of microtubules. The major soluble protein extracted by the treatment has been extensively compared with outer doublet microtubule protein from sea urchin sperm flagella, which was extracted by similar treatment. The fact that outer doublet microtubule protein from sperm flagella is now known to be a mixture of two similar proteins, *a*-tubulin and *b*-tubulin (36, 37), has not in practice interfered with the analysis; indeed, it appears that the protein from mitotic apparatus is also composed of two closely related species.

MATERIALS AND METHODS

Biological

Sea urchins, *Strongylocentrotus purpuratus*, were obtained from Pacific Bio-Marine Supply Co., Venice, Calif. Gametes were obtained by injection of 0.5 M potassium chloride, and handled at 15°–17°C in artificial seawater. Fertilization membranes were removed as described previously (31). Mitotic apparatus was isolated at first metaphase by the method of Kane (38), using molar 2-methyl-2,4-pentanediol in 0.01 M maleate buffer, pH 6.4. Further handling of all preparations was done at 4°C. Isolated mitotic apparatus was washed four times by centrifugation (3 min at 600 *g*) and resuspension in isolation medium, and collected by a final centrifugation of 5 min at 600 *g*. Sperm were processed according to Stephens et al. (14) to obtain outer doublet microtubule preparations.

Electron Microscopy

Three fixation procedures were used. The first (H. Sato, personal communication) is known to preserve birefringence of isolated mitotic apparatus. Samples were placed at 15°C in 3% glutaraldehyde and 12% 2-methyl-2,4-pentanediol in 0.01 M phosphate buffer, pH 6.5, and cooled to 4°C. After washing, samples were postfixed in 2% osmium tetroxide in the same medium. Dehydration was begun in ethanol diluted to 30% with the same medium. The second fixation procedure was similar, but fixation and postfixation were combined, that is, fixation was carried out in 2% osmium tetroxide, 3% glutaraldehyde, and 12% 2-methyl-2,4-pentanediol in 0.01 M phosphate buffer, pH 6.5. This gave very satisfactory preservation of untreated mitotic apparatus. The third fixative, and the only one used for outer doublet microtubule preparations, was 2% osmium tetroxide in acetate buffer isotonic with seawater, pH 6.1 (39). Samples were cooled to 4°C in fixative, and transferred directly to 70% ethanol. Further dehydration was carried out in graded alcohols and propylene oxide. Embedding was done in Epon 812 (40). Sections were cut on an LKB Ultrotome III (LKB Instruments, Inc., Rockville, Md.), stained for 2 hr with 7% aqueous uranyl magnesium acetate (W. Stoeckenius, personal communication) and briefly with lead citrate (41), and examined in a Hitachi HU-11B electron microscope.

Protein Determination

The method of Lowry et al. (42) was used for protein determinations, except for those made in the presence of vinblastine which required the use of the biuret method (43).

Polyacrylamide Gel Electrophoresis

Two systems were employed, one acid and one basic, both containing urea. The acid system was that of Takayama et al. (44, 45). Pre-electrophoresis of the gels was carried out with polarity reversed, to remove ammonium persulfate; the gel tubes were filled with the same acetic acid and urea solution contained in the gels, and the electrode chambers contained the same solution without urea. Ribonuclease was used as a marker. The basic system was that of Davis and Ornstein (46, 47), except that 8 M urea was used instead of water in making up the gels. Spacer and sample gels were omitted, and samples, made up in the buffer normally used for the sample

gel but containing 8 M urea, were layered on the gels. Pre-electrophoresis was carried out to remove ammonium persulfate, using in the gel tubes the same buffer contained in the gels, and in the electrode chambers the same buffer without urea.

Gels were fixed in 12% trichloracetic acid, stained in Coomassie Blue, (Canalco Inc., Rockville, Md.), and destained in 7% acetic acid. Storage in 7% acetic acid resulted in further destaining over several months, and was often required to reveal doublet bands.

Relative mobilities were measured by projecting images of the gels in a photographic enlarger. The migration distances of bands and markers were obtained as the average from three measurements.

Molecular Weight Determination

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate containing gels was carried out according to Shapiro et al. (48), using 5% gels and 0.01 M buffer in gels and electrode chamber, or according to Weber and Osborn (49) using 10% gels. Proteins used as references were egg white lysozyme, bovine hemoglobin, chicken ovalbumen, and bovine serum albumen from Pentex Biochemical, Kankakee, Ill.; bovine pancreatic chymotrypsinogen-A from Mann Research Labs Inc., New York, and bovine pancreatic DNase-1 from Sigma Chemical Co., St. Louis, Mo. Molecular weights were obtained from references 49 and 50.

Immunological Procedures

Two different immunization procedures were used, and only the second produced active antiserum to microtubule protein. Antigens for the first procedure were meralluride extracts (high speed supernatant) of sperm tail outer doublet microtubules and isolated mitotic apparatus. The antigens were dialyzed against 0.15 M sodium chloride in 0.01 M phosphate buffer, pH 7.3, and about 0.5 mg of protein was injected into the cornea of rabbits. Seven intramuscular injections of about 2 mg each were then given at 2-day intervals. 4 wk later, a further 0.5 mg was injected corneally. The first bleeding was done a week later. For the second procedure, hyperimmunizing conditions (high doses of antigen) were used. The antigen was intact sperm tail outer doublet microtubules, selected for morphological purity by electron microscopy. Approximately 50 mg was homogenized in buffered saline (see above) and Freund's complete adjuvant (Difco Laboratories, Inc., Detroit, Mich.) and injected intramuscularly into rabbits. The injection was repeated after 4 and 10 wk. The first bleeding was done 2½ wk thereafter. Subcutaneous injections of 5 mg of antigen were given 5 days before later bleedings.

Immunoelectrophoresis and other tests to determine that this antiserum was not merely against impurities in the microtubule preparation are described below (see Results).

Immunodiffusion experiments were done by Ouchterlony's technique (51), using 1% agarose. Immunoelectrophoresis was carried out in 0.5% agarose in barbital buffer at pH 8.6 (see reference 31 for details).

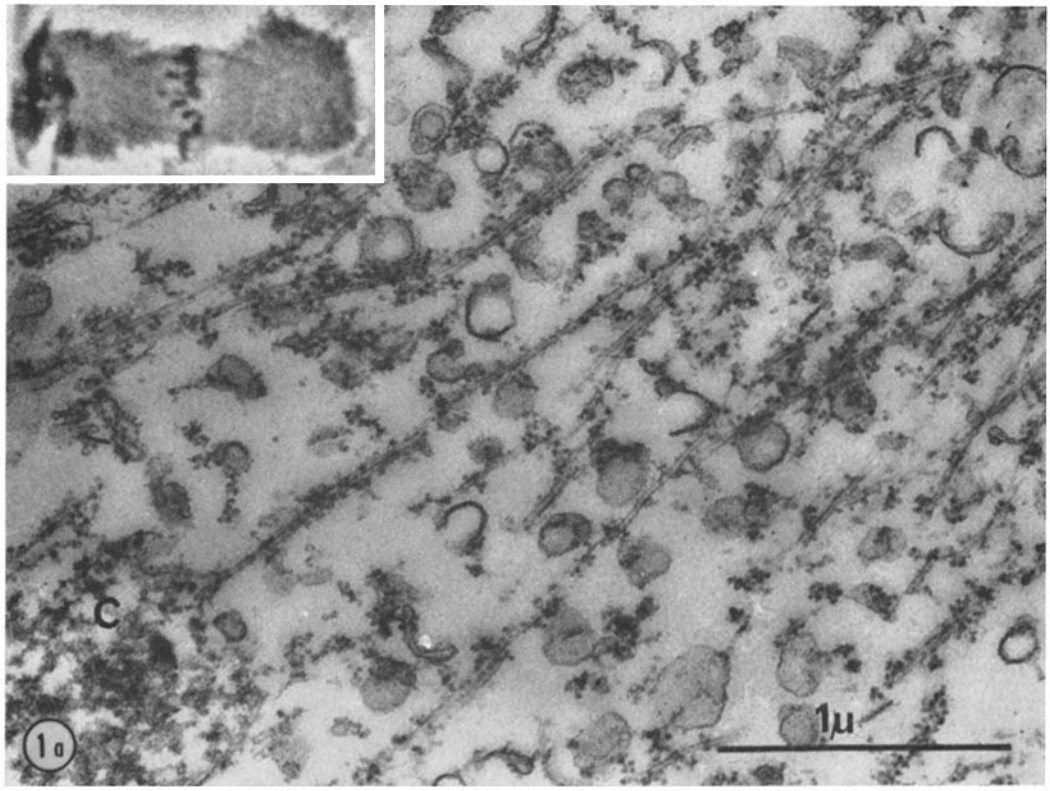
Amino Acid Analysis

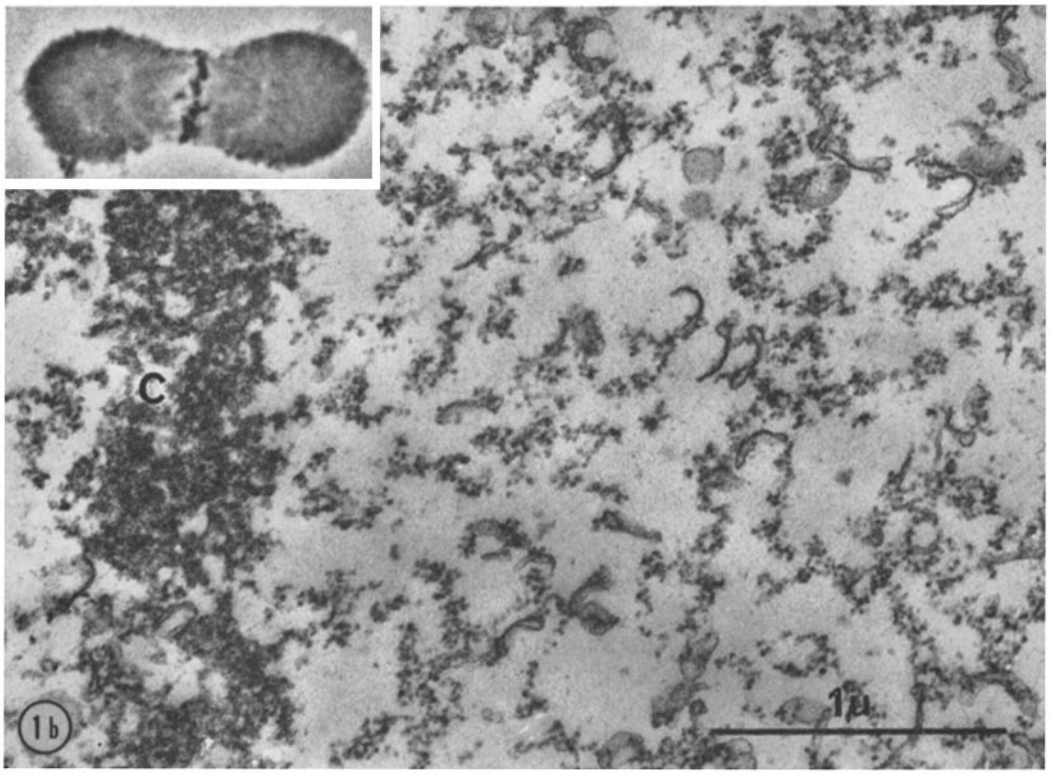
After dialysis of the sample against 0.01 M borate, pH 9.0, amino acid analysis was performed by the method of Moore and Stein (52). The sample was hydrolyzed in 6 N hydrochloric acid in an evacuated, sealed Pyrex tube at 110 ± 1°C for 20 hr. After removal of the hydrochloric acid by rotary evaporation, the analysis was performed with the automatic analyzer system of Spackman, Stein, and Moore (53) on a Beckman-Spinco Model 120C amino acid analyzer (Beckman Instruments, Fullerton, Calif.) with an Infotronics Model CRS-12-AB integrator (Infotronics, Inc., Houston, Texas). Ninhydrin was made up according to Moore (54) with dimethyl sulfoxide as solvent.

Sedimentation Analysis

Sedimentation analysis was done in a Beckman-Spinco Model E analytical ultracentrifuge, using

FIGURE 1 (*a*) Appearance of washed, isolated mitotic apparatus, treated 1 hr with 0.013 M theophylline in 0.01 M maleate buffer, pH 6.5, as a control for extraction with meralluride. Fixation in 2% osmium tetroxide buffered at pH 6.1. A region of the spindle near the chromosomes (*C*) is shown. Microtubules are evident, as well as ribosomes and membranous vesicles. (*b*) Washed isolated mitotic apparatus from the same experiment, after extraction with 0.01 M meralluride sodium in 0.01 M maleate buffer, pH 6.5, for 1 hr. Fixation as in (*a*). Microtubules are entirely absent, but the morphology is otherwise well preserved. Chromosomes (*C*), ribosomes and vesicles are unaltered. *Insets*: 4-μ sections of each mitotic apparatus cut immediately before sectioning for electron microscopy, showing that the thin sections are approximately median and longitudinal. This assures that every thin section will contain microtubules when they are present in the specimen. Fig. 1 *a*, ×39,000; Fig. 1 *b*, ×39,000.


C
1μ
1a


C
1μ
1b

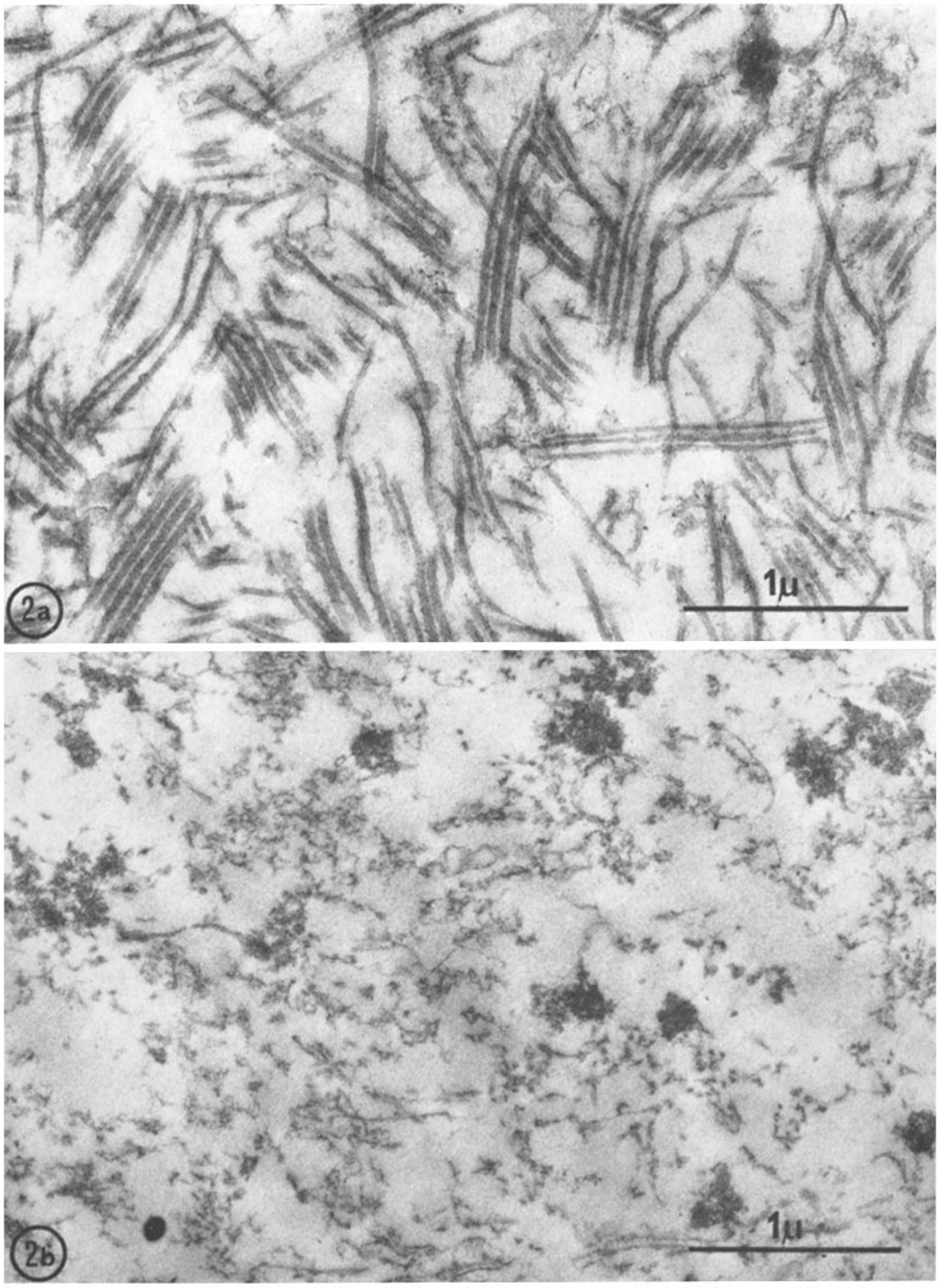


FIGURE 2 (*a*) Sectioned pellet of a preparation of outer doublet microtubules from sperm flagella. (*b*) A section of a pellet from the same outer doublet microtubule preparation after extraction of 65% of the protein with 0.01 M meralluride in 0.01 M borate buffer, pH 9.0. No microtubules are present, and visible features are evidently contaminants in the original preparation. Fig. 2 *a*, ×33,500; Fig. 2 *b*, ×33,500.

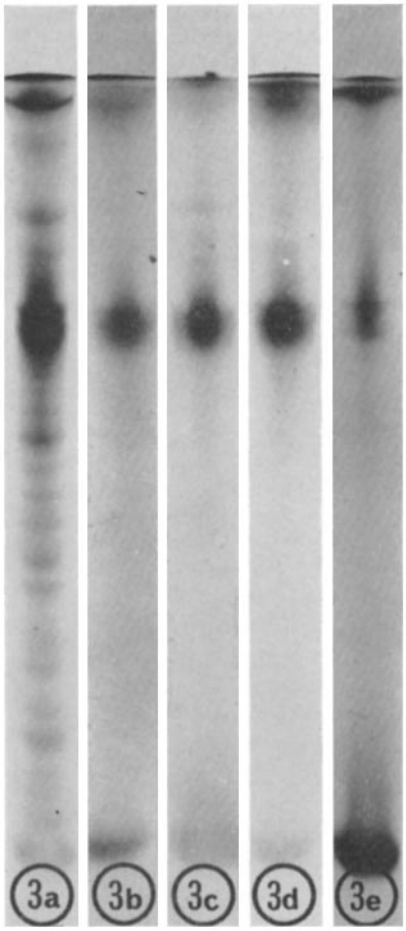


FIGURE 3 Polyacrylamide gel electrophoresis of meralluride extracts of isolated mitotic apparatus and of outer doublet microtubules from sperm flagella, in the acid, urea-containing system of Takayama et al. (44). The bottom band in each gel is ribonuclease, used as marker. Gels were enlarged as required to obtain alignment of the markers. (*a*) Meralluride extract of isolated mitotic apparatus (low speed supernatant). Particulates removed from the extract by 2 hr centrifugation at 17,000 rpm (Sorvall SS-34 rotor). The band near the top of the gel is "22S protein." (*b*) Meralluride extract of isolated mitotic apparatus (high speed supernatant). Particulates removed by 4 hr centrifugation at 50,000 rpm (Spinco SW 50L rotor). (*c*) Meralluride extract of outer doublet microtubules from sperm flagella. Centrifugation as in (*b*). Note absence of a 22S protein band. (*d*) Calcium precipitate of meralluride extract of isolated mitotic apparatus, redissolved to volume in 8 M urea. (*e*) Vinblastine precipitate of meralluride extract of mitotic apparatus, redissolved to volume in 8 M urea. In this case, the protein near the top of the gel is not in the migration position for 22S protein, but has imperfectly penetrated the gel. The major doublet shows a small change in mobilities which is probably real.

schlieren optics. Because we were not able to concentrate the meralluride extract of mitotic apparatus without precipitating the protein, we were obliged to use a protein concentration of approximately 1 mg/ml. Centrifuge cells with 30 mm pathlength were used in some determinations.

RESULTS

Extraction of Mitotic Apparatus and Outer Doublet Microtubules by Meralluride

MORPHOLOGICAL EFFECTS: Isolated washed mitotic apparatus was recovered as a pellet and resuspended in an approximately equal volume of a meralluride sodium solution, consisting of Mercuhydrin (Lakeside Laboratories, Milwaukee, Wis.) diluted to 0.01 M with 0.01 M maleate buffer, pH 6.5. The suspension was allowed to stand for 1 hr at 4°C. After this treatment, mitotic apparatus remains intact, but examination in a polarizing microscope shows that the birefringence is almost totally lost. Electron microscopy, with use of three different fixatives, shows that the microtubules have disappeared, but that the characteristic morphology of isolated mitotic apparatus is otherwise well preserved (Fig. 1 *b*). Microtubules do not reappear if extracted mitotic apparatus is returned to isolation medium for an hour before fixation.

The disappearance of microtubules specifically requires the presence of meralluride. To test the stability of microtubules in diluted isolation medium per se, mitotic apparatus was treated as above with maleate buffer alone. A further control was required because Mercuhydrin as supplied by Lakeside Laboratories contains theophylline, which we made no effort to remove. Accordingly, mitotic apparatus was also treated with maleate buffer containing 0.013 M theophylline, an amount equal to that present in experimentals. Neither control showed loss of birefringence after treatment, and both contained microtubules when examined in the electron microscope (Fig. 1 *a*). (It should be mentioned that we have not generally observed as rapid a loss of birefringence or microtubules in washed mitotic apparatus as was observed by Goldman and Rebhun [35].)

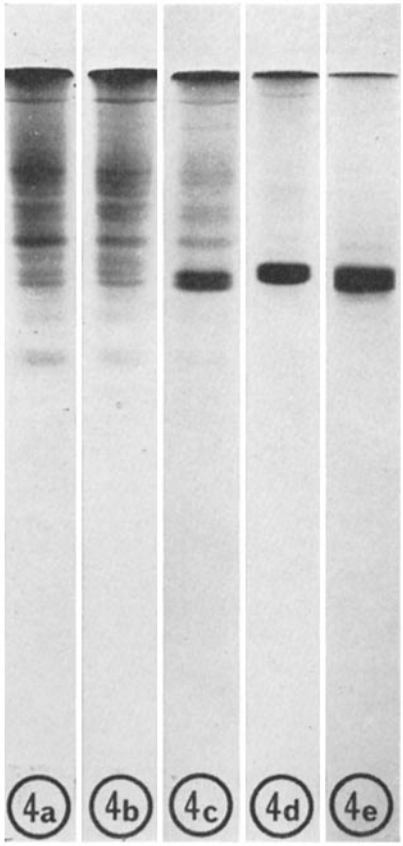


FIGURE 4 Polyacrylamide gel electrophoresis of meralluride extract of isolated mitotic apparatus and sperm tail outer doublet microtubules in the basic polyacrylamide system of Ornstein and Davis (46, 47) containing 8 M urea, and comparison of protein extracted from mitotic apparatus with protein extract from nonmitotic apparatus particulates. Gels were variably enlarged to align the migration positions of a bromphenol blue marker (bottom edge of figure). (*a*) Meralluride extract (low speed supernatant) of nonmitotic apparatus particulates from the "first supernatant" obtained during isolation of mitotic apparatus (see text). (*b*) Meralluride extract (low speed supernatant) of nonmitotic apparatus particulates from the "second supernatant." (*c*) Meralluride extract of isolated mitotic apparatus (low speed supernatant). (*d*) Meralluride extract of isolated mitotic apparatus (high speed supernatant). (*e*) Meralluride extract of outer doublet microtubules from sperm flagella. Centrifugation as in (*d*). Note the absence of a faint contaminant band near the top of the gel which is characteristic of mitotic apparatus.

Extraction with meralluride also causes the selective disappearance of sperm tail "outer doublet" microtubules. When a preparation of outer doublet microtubules (Fig. 2 *a*) is treated with meralluride, and the insoluble fraction is sedimented, as described previously (27), electron microscopy of the insoluble fraction shows it to consist of contaminants, such as unit membrane fragments, which were present in the original preparation. No microtubules are visible (Fig. 2 *b*).

EXTRACTION OF PROTEIN: After incubation to extract mitotic apparatus and centrifugation to remove particulates, the supernatant was examined for extracted protein. In preliminary experiments, centrifugation was done for 2 hr at 17,000 rpm in the Sorvall SS-34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.). This supernatant contained about 20% of the protein of mitotic apparatus, while re-extraction of the pellet recovered only an additional 4%, showing that extraction was largely complete with a single treatment. The polyacrylamide gel electrophoresis pattern of this supernatant (Fig. 3 *a* and 4 *c*), while showing more heterogeneity than expected from the morphological results, had an unambiguous major band (actually a doublet, as will be discussed below). Solubilization of the major protein depended on the presence of meralluride, since the major doublet was absent if extraction was carried out with buffer only or buffer with theophylline (only 2–3% of mitotic apparatus protein was extracted in these controls). The major doublet did not reappear in control extracts if meralluride was added before electrophoresis.

In further work, centrifugation after extraction was done for 4 hr at 50,000 rpm in the Spinco SW 50L rotor (Beckman Instruments). When this higher speed centrifugation is used, the protein recovered in the supernatant drops to about 10% of the protein of isolated mitotic apparatus, and a remarkable simplification of the polyacrylamide gel electrophoresis pattern results (Figs. 3 *b* and 4 *d*). All bands except the major doublet are either removed, or reduced to trace amounts. The extent to which the pattern is simplified suggests that many of the proteins of the low speed supernatant are actually present in cellular particulates, which are only later dispersed by the urea medium used for electrophoresis (cf. reference 29). Any proteins rendered truly soluble at the time of extraction should still be present in the high speed supernatant, except for those which (like 22S protein)

have molecular weights greater than several hundred thousand.

The high speed supernatant constitutes the "meralluride extract" routinely employed in the work to be described. For symmetry, the same centrifugation was used to remove particulates from meralluride extracts of sperm tail outer doublet microtubules.

Properties of the Extracted Major Protein

ELECTROPHORETIC MOBILITY: Electrophoretic mobilities were determined in acid and basic polyacrylamide gel systems (see Methods). In the basic system the extract of mitotic apparatus may at first appear to give a wide major band (r_f approximately 0.27) but when only a small quantity of protein is used, or after extensive destaining, the band is revealed to be a doublet. The r_f values of the individual bands are approximately 0.267 and 0.281 (averages from four gels, each from a separate experiment). In the acid system the region of dense staining always consists of two bands, with relative mobilities with respect to ribonuclease of approximately 0.313 and 0.332 (averages from four gels, as above).

The meralluride extract of outer doublet microtubules of sperm tail gives major bands virtually identical to those of mitotic apparatus in both acid and basic gels (Figs. 3 *c*, 4 *e*). In basic gels, and under the same conditions, there is either a single band (r_f value approximately 0.27) or a doublet with r_f values for the individual bands close to or possibly equal to those of the mitotic apparatus doublet (values of 0.264 and 0.278 were obtained as the averages from four gels, as above). In acid gels, the major band is always a doublet, with relative mobilities again close to or equal to those of mitotic apparatus protein (approximately 0.312 and 0.334, averages from six gels). When a mixture containing equal amounts of protein from extracts of mitotic apparatus and outer doublet microtubules is subjected to electrophoresis in the acid system, a doublet band still forms and is indistinguishable from those of the proteins run separately.

PRECIPITATION BY CALCIUM AND VINBLASTINE: Microtubule proteins characteristically precipitate in the presence of calcium (27, 55) and vinblastine (7–9, 11–13). When the extract of mitotic apparatus is made 0.05 M in calcium ion at 4°C, a precipitate forms slowly. Prompt precipitation can be induced by warming slightly, while warming alone does not cause precipitation. The precipitate contains 80–85% of the protein of the extract. It can be redissolved in 8 M urea, and its polyacrylamide gel electrophoresis pattern then gives the characteristic major bands of the meralluride extract (Fig. 3 *d*). These bands are also present, but considerably diminished, in the calcium soluble fraction, while a number of faint bands are most intense in this fraction.

When vinblastine sulfate (Velban, Eli Lilly and Co., Indianapolis, Ind.) from a solution previously titrated to pH 6.5 is added to the mitotic apparatus extract to a concentration of 0.004 M, a heavy precipitate forms immediately. Protein determinations by the biuret method indicate that more than 90% of the protein is precipitated. The precipitate, redissolved in 8 M urea, gives a major doublet band on polyacrylamide gel electrophoresis, while the vinblastine-soluble fraction contains only contaminant bands, with no trace of the major doublet. Precipitation of the major protein thus appears to be quantitative. The doublet given by the vinblastine precipitate is not identical to the doublet before treatment. In both acid and basic systems, there appears to be a slight but significant shift in mobilities (Fig. 3 *e*).

Control experiments on precipitation by vinblastine show that the concentration used precipitates neither 22S protein from sea urchin eggs nor bovine serum albumen.

Tests of precipitation by calcium and vinblastine carried out on the meralluride extract of sperm tail outer doublet microtubules show a behaviour closely similar to that of the mitotic apparatus extract. The percentages of precipitation are similar, and polyacrylamide gel electrophoresis of the redissolved precipitate gives major doublets similar to the ones which are characteristic of the extract.

SUBUNIT MOLECULAR WEIGHT: Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (48, 49, 56) was used to estimate the molecular weight, after treatment with sodium dodecyl sulfate and mercaptoethanol, of the major proteins of the extracts of mitotic apparatus and sperm tail outer doublet microtubules. Three determinations were made, each including electrophoresis of several replicate samples.

This system, which discriminates molecular weights, does not resolve doublets in either the mitotic apparatus or the sperm tail protein, indicating that in each case the protein making up the

two bands of the doublet is uniform in molecular weight. The mobilities of the mitotic apparatus and sperm tail proteins are closely similar; in our determinations their average r_f values, calculated from 10 gels for each protein, differed by only 0.2% of their value. Mixtures of equal parts of the two proteins gave a band identical in appearance and mobility to the bands of either protein alone. The estimated molecular weight, averaged from all determinations, was 52,000 for both proteins, in reasonable agreement with values of 56,000–66,000 previously reported for outer doublet microtubule protein from sea urchin sperm (5, 17, 19).

SEDIMENTATION ANALYSIS: After dialysis against 0.01 M meralluride in 0.01 M maleate buffer, pH 6.5, the extract of mitotic apparatus at a concentration of about 1 mg protein per ml (see Methods) gave a boundary with a sedimentation coefficient (S_{20}) of 8.9S. This is close to the value obtained by Stephens (17) for outer doublet microtubule protein extracted by low concentrations of mersalyl at pH 8.5. After dialysis against 0.01 M meralluride in 0.01 M borate buffer at pH 9.0, a different sedimentation coefficient, 4.9S, was obtained. This again is very close to the value for the same concentration of protein for outer doublet microtubule protein extracted by higher concentrations of mersalyl (0.05 M) at pH 8.5. The two sedimentation coefficients appear to correspond to two association states of the proteins. Each protein displays two states in solutions of mercurial, and, as indicated by closely similar sedimentation coefficients, each state of one protein corresponds to a state of the other.

To obtain a sedimentation profile of the mitotic apparatus protein at higher concentration, the protein was precipitated by calcium or by reduction of solvent by dialysis against polyvinyl pyrrolidone, and was redissolved in urea. In this form, it still gives a single boundary (Fig. 5); the protein making up the electrophoretic doublet is too similar in sedimentation rate to be resolved. The same is true (17, 37) for outer doublet microtubule protein from sperm flagella.

AMINO ACID COMPOSITION: Our data on the amino acid composition of the mitotic apparatus protein are preliminary. A determination has been made of the amino acid composition of the meralluride extract from mitotic apparatus (high speed supernatant). Tryptophan and cysteine have not been determined. However, the available data have substantial similarity to the amino acid composition of known microtubule proteins, as can be seen in Fig. 6.

IMMUNOCHEMICAL RELATIONSHIP OF THE MITOTIC APPARATUS AND SPERM TAIL PROTEINS: An antiserum has been obtained against intact outer doublet microtubules. When tested by immunodiffusion or immunoelectrophoresis against the meralluride extract of these microtubules, the antiserum gives two or three precipitin bands. One of the antigens responsible for these bands apparently migrates electrophoretically with the same mobility as the major protein of the extract (Fig. 8 *b*, *c*, *d*).

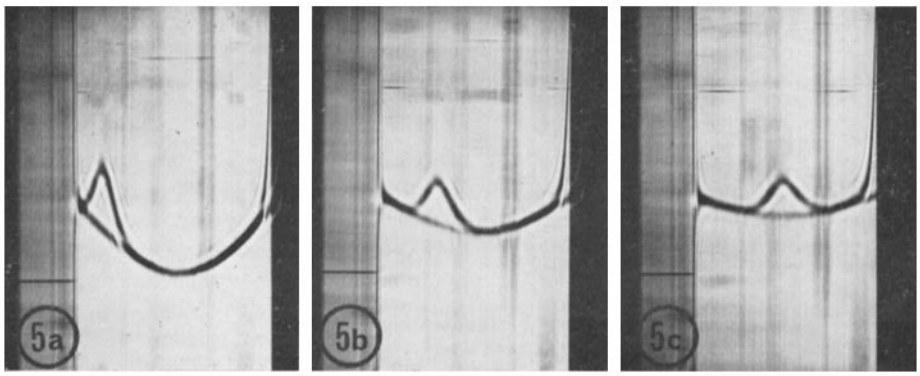


FIGURE 5 Analytical ultracentrifugation of the meralluride extract of mitotic apparatus in 8 M urea. The extract was precipitated by 0.05 M calcium ion, and redissolved in 8 M urea at a concentration of 4.2 mg protein per ml. The run was performed at 20°C and 60,000 rpm. Analyzer angle 50°. Times after reaching 40,000 rpm are (*a*) 138 min, (*b*) 266 min, (*c*) 394 min.

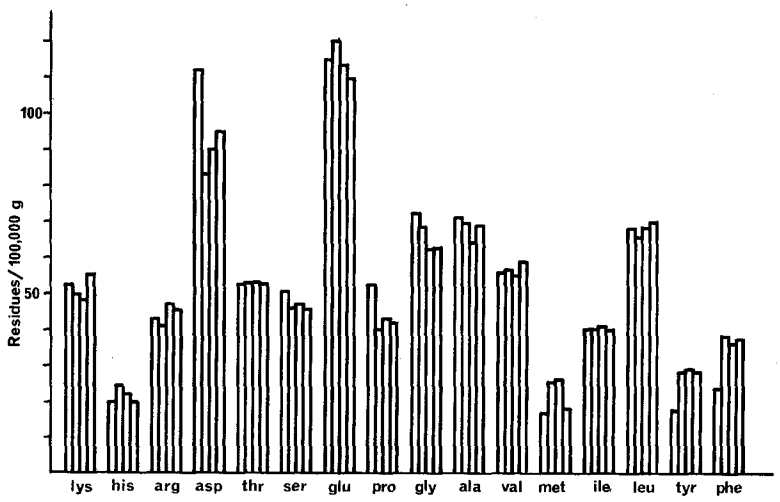


FIGURE 6 Comparison of preliminary data on amino acid composition of the meralluride extract of isolated mitotic apparatus with the amino acid compositions of outer doublet microtubule proteins from sperm flagella of three species of sea urchin. The residues per 100,000 g of the 16 amino acids have been plotted. The first bar of each group represents meralluride extract from mitotic apparatus. Second bar: outer doublet microtubule protein from *Strongylocentrotus purpuratus* (reference 5). Third bar: from *Strongylocentrotus droebachiensis* (reference 17). Fourth bar: from *Anthocidaris crassispina* (reference 15). The data for meralluride extract of mitotic apparatus are *lys*, 52.5; *his*, 19.8; *arg*, 42.9; *asp*, 112.0; *thr*, 52.5; *ser*, 50.6; *glu*, 114.8; *pro*, 52.3; *gly*, 72.1; *ala*, 70.9; *val*, 55.8; *met*, 16.7; *ile*, 40.1; *leu*, 68.0; *tyr*, 17.6; *phe*, 23.6.

When the meralluride extracts of outer doublet microtubules and of mitotic apparatus are tested by immunodiffusion against the antiserum, a single precipitin band forms against the mitotic apparatus extract and fuses with one of the bands given by the sperm tail extract. The band is weaker on the mitotic apparatus side when equal concentrations of the extracts are placed in the wells, but there is no spur as such (Fig. 7).

An immunoelectrophoresis experiment with meralluride extract of mitotic apparatus as antigen shows that the antigen responsible for the cross-reaction has the same electrophoretic mobility as the major protein of the extract (Fig. 8 *a*). Another immunoelectrophoresis experiment in which the sperm tail extract is used as antigen, but in which the formation of a precipitin band by the cross-reacting antigen is blocked by prior exposure of the antiserum to mitotic apparatus extract, shows that the cross-reacting antigen in the sperm tail extract is identical to the antigen having the same electrophoretic mobility as the major protein of that extract (Fig. 8 *e*).

Further evidence that the cross-reacting antigens in the two extracts are identical to their major pro-

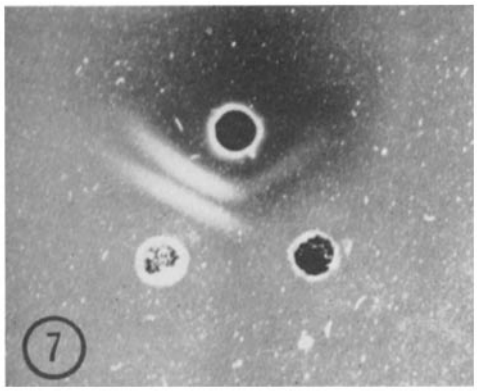


FIGURE 7 Immunodiffusion test of the cross-reactivity of meralluride extract (high speed supernatant) from isolated mitotic apparatus and extract of sperm tail outer doublet microtubules. Top well: antiserum to a preparation of intact outer doublet microtubules. Left well: meralluride extract of outer doublet microtubules. Right well: meralluride extract of isolated mitotic apparatus. The preparations in the two antigen wells are at the same concentration (0.64 mg/ml). The extract from mitotic apparatus gives a single band, which merges with a band given by extract from outer doublet microtubules.

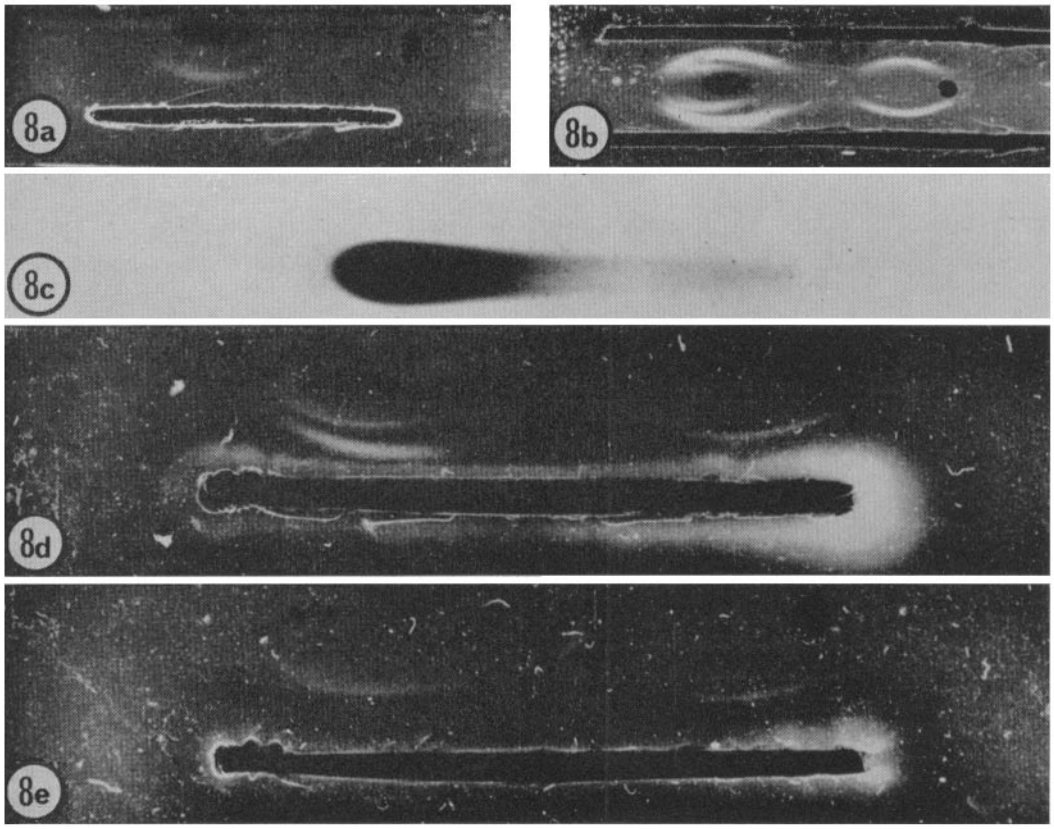


FIGURE 8 Immunoelectrophoresis of meralluride extracts of mitotic apparatus and sperm tail outer doublet microtubules, using antiserum against a preparation of outer doublet microtubules. (*a*) Antigen: meralluride extract of mitotic apparatus. Electrophoresis was carried out on twin plates, in parallel. One plate was fixed and stained with Coomassie Blue, the other was reacted with antiserum placed in the trough. In this figure, photographs of the two plates have been superimposed, with the sample wells in register. The dark area represents the stain, and shows the position of the major protein after electrophoresis. A precipitin band brackets this position, showing that an antigen migrating with the mobility of the major protein cross-reacts with antiserum to outer doublet microtubules. (*b*) Antigen: meralluride extract of outer doublet microtubules. Procedure as in (*a*), but antiserum was placed in two troughs, top and bottom. Three precipitin bands are demonstrable. The strongest band has a good correspondence to the position of the major protein. (*c*) and (*d*) A repeat of the experiment shown in (*b*). The stained plate (*c*) and the plate used for immunodiffusion (*d*) are shown separately. The strongest band again corresponds well in position with the major protein after migration. (*e*) A plate with extract of outer doublet microtubules as antigen run concurrently with the plates shown in (*c*) and (*d*), and identical with (*d*), except that the antiserum placed in the trough had previously been absorbed with extract from mitotic apparatus to remove antibodies responsible for the cross-reaction. After absorption, the precipitin band corresponding to the major protein fails to form.

teins comes from the finding that pretreatment of either extract with antiserum, and removal of the resulting precipitates, eliminates the ability of the extracts to yield a precipitate on addition of calcium. For this experiment, samples of antiserum and extract were dialyzed against 0.15 M sodium chloride buffered with Tris-HCl (0.01 M, pH 7.5) and mixed in various proportions. Antigen-antibody precipitation was visible within minutes; incubation was continued for 20 min at 37°C and 6 hr at 4°C. No precipitate formed in control samples of the extracts (buffered saline added instead of antiserum). Samples were centrifuged to sediment any precipitate that was present, and

the upper portion of fluid was removed; calcium chloride was added to the fluid to a final concentration of 0.05 M. A precipitate formed in the control samples, and accounted for approximately 40% of the extract protein which was added at the beginning of the experiment. This is a low value for precipitation by calcium, but the proteins from the extracts were present in low concentration, precipitation may be partially inhibited by sodium, or some protein aggregates may have formed during the control incubation and been removed by centrifugation prior to the addition of calcium. No precipitate formed on addition of calcium to the experimental samples containing the highest ratios of antiserum to extract (antibody excess); raising the level of calcium to 0.1 M still gave no precipitate. A small precipitate, containing less than 40% of the extract protein, formed in the samples containing the highest ratios of extract to antiserum (antigen excess); this shows that the presence of serum per se does not prevent precipitation.

All tests of the proposition that the major proteins of the extracts are identical to the cross-reacting antigens have so far given positive results, and we provisionally conclude that the major protein of the meralluride extract of mitotic apparatus cross-reacts with microtubule protein from sperm tail outer doublets. Fulton, Kane, and Stephens (32, 57) have obtained similar results: a cross-reaction between extracts from mitotic apparatus considered to contain microtubule protein and antibody against outer doublet microtubules from sea urchin sperm flagella.

Specific Location in Mitotic Apparatus of the Major Protein Extracted by Meralluride

The possibility of contamination of isolated mitotic apparatus by proteins not properly a part of it has been discussed by many workers (24, 29, 31, 58). No simple test can determine whether a protein recovered from isolated mitotic apparatus is a functional part of it, but it is possible to distinguish between proteins specifically located in mitotic apparatus and those which are distributed over all cellular particulates under the conditions of isolation. We have examined cellular particulates other than mitotic apparatus for the presence of the protein extracted by meralluride. The following fractions were collected during isolation of mitotic apparatus (38): the "first supernatant," containing substances released into the first change of isolation medium by the swollen eggs; the "second supernatant," containing the substances remaining after dispersal of the eggs and collection of mitotic apparatus by centrifugation; and a pellet of unwashed mitotic apparatus. Particulates were recovered from the first and second supernatants by centrifugation (30 min at 17,000 rpm in the Sorvall SS-34 rotor), and the resulting pellets, as well as the mitotic apparatus pellet, were suspended in approximately equal volumes of mitotic apparatus isolation medium. To each suspension, an equal volume of 0.01 M meralluride in 0.01 M maleate buffer, pH 6.5, was then added. After an hour of extraction, the samples were centrifuged for 2 hr at 17,000 rpm in the Sorvall SS-34 rotor (this is the lower speed centrifugation of those we have used to remove particulates from meralluride extracts, and its use here facilitates the study of contaminants). Equal samples of each supernatant were analyzed by polyacrylamide gel electrophoresis in the basic system. The extract of mitotic apparatus showed the characteristic doublet band of the major protein (Fig. 4 *c*), as well as typical contaminant bands. In the other extracts, the contaminant bands were much stronger, while the doublet was much weaker (Fig. 4 *a*, *b*). These results show that much less of the major protein is recoverable per unit volume from nonmitotic apparatus particulates than from mitotic apparatus. The same result holds per milligram of protein of the particulates. The extracted major protein clearly forms a much smaller percentage of protein extracted from the nonmitotic apparatus particulates than is the case for mitotic apparatus. In addition, protein determinations show that a smaller percentage of total protein is extracted in the case of the nonmitotic apparatus particulates, so that the major protein extracted forms a still smaller percentage of their total protein. The major protein, in a form extractable by meralluride, is thus considerably more concentrated in mitotic apparatus than in other particulates.

DISCUSSION

Extraction of isolated mitotic apparatus by the organic mercurial meralluride, under conditions otherwise kept close to those of its isolation, causes the selective morphological disappearance of microtubules. Protein is extracted by this treatment which has extensive similarities to outer doublet microtubule protein from sperm flagella.

These similarities include its extraction under conditions which depolymerize the sperm tail microtubules; closely similar electrophoretic mobilities in both acid and basic polyacrylamide gels containing urea; closely similar molecular weights; precipitation by calcium ion and vinblastine; similarity in sedimentation coefficient in two distinct states of aggregation, and similarity in amino acid composition. Moreover, the protein appears to cross-react immunochemically with antibody against microtubule protein from sperm tail. Such cross-reaction, more particularly than any of the other findings, indicates homology between the proteins, i.e., substantial similarities in amino acid sequence and tertiary structure. Similarities of the kind described are to be expected among microtubule proteins. The data are therefore entirely consistent with the interpretation that the major protein of the meralluride extract of mitotic apparatus is microtubule protein, and is typical in its properties.

Our results are consistent with data from other laboratories which show resemblances between protein from mitotic apparatus and typical microtubule proteins. Borisy and Taylor (26) have described a colchicine-binding protein in sea urchin eggs and in isolated mitotic apparatus. The 6S sedimentation coefficient of the protein, measured in a sucrose gradient, is equal to the sedimentation coefficient of other colchicine-binding proteins and microtubule proteins obtained in that laboratory. Stephens (17) has mentioned studies of amino acid composition of a protein fraction from the potassium chloride extract of isolated mitotic apparatus, and reports that its amino acid composition is almost identical to that of sperm tail outer doublet microtubule protein. Fulton, Kane, and Stephens (32, 57) have shown that antibody against outer doublet microtubules from sperm flagella cross-reacts with a protein fraction from mitotic apparatus considered to contain microtubule protein.

In the case of preparations of outer doublet microtubules from sperm tail, the proposition that the major protein extracted by meralluride is microtubule protein is convincing primarily because one cannot imagine that another protein can account for the amount of major protein extracted. In our preparations, 65% of the total protein is extracted, mostly as major protein. Stephens (17), however, reports extraction of more than 90% of the protein of an outer doublet preparation from *Strongylocentrotus droebachiensis* by the organic mercurial mersalyl, and the major protein accounts for almost all of the protein extracted.

Because of the complexity of mitotic apparatus, a comparable argument cannot be made, but the amount of protein we obtain is probably very similar to the amount of microtubule protein present in the system. If taken as the amount of protein in the high speed supernatant it is 10% of the protein of isolated mitotic apparatus, or if taken as the amount of calcium or vinblastine precipitable protein it is 8–9% of mitotic apparatus protein. Cohen and Rebhun (59) have counted the profiles of microtubules in isolated mitotic apparatus, and can calculate that 6–12% of mitotic apparatus protein is microtubule protein. Borisy and Taylor (26) have calculated that 5–15% of the protein of isolated mitotic apparatus is colchicine-binding protein.

The fact that mitotic apparatus is a much richer source of the major protein extracted by meralluride than are other particulates is again consistent with the contention that the major protein is microtubule protein. Its distribution appears to coincide with the distribution of intact microtubules. In particular, the protein is not a normally soluble contaminant of mitotic apparatus that is artifactually present because of limited solubility under conditions of isolation and extracted because of greater solubility in the extraction medium; little of the protein is likely to consist of normally soluble microtubule precursor proteins, including ciliary precursor. Although such soluble precursors would have properties very similar to those of sperm tail microtubule protein, they would be expected to distribute themselves equally among the various particulates during mitotic apparatus isolation. The specific requirement for meralluride to achieve extraction of the major protein supports this point.

Several important characteristics of microtubule protein remain to be demonstrated for the protein extracted from mitotic apparatus by meralluride. We anticipate that the protein can be shown to be identical with the colchicine-binding protein recovered from mitotic apparatus by Borisy and Taylor (26), and that it can be shown to contain one guanine nucleotide per monomer molecule, at least in its native form. By analogy with outer doublet microtubule pro-

tein extracted by mercurial (17), the form of the mitotic apparatus protein having a sedimentation coefficient near 5S is probably a dimer of the unit whose molecular weight we estimate as 52,000.

The finding that proposed microtubule protein from mitotic apparatus gives a double band on electrophoresis in polyacrylamide gels was unexpected. Moreover, each component band has an electrophoretic mobility closely similar to that of a corresponding band of outer doublet microtubule protein. Doublet bands have heretofore been described for outer doublet microtubule protein from cilia and flagella (16, 37, cf. also 36), and have been attributed to nonidentical proteins of the *a*-tubule and *b*-tubule, respectively. Two speculations are suggested by the present finding. First, there are probably two chemically distinct kinds of microtubule in mitotic apparatus, as for example chromosomal and continuous microtubules. Second, these two kinds of microtubule may be functionally related to the *a*- and *b*-tubules, respectively, of outer doublets of ciliar and flagella. In these doublets only the *a*-tubule holds the arms, composed of the ATPase dynein, which are considered to be the site of the mechanochemical act during motility (60–62). Arms and crossbridges have recently been described also in mitotic apparatus (63, 64), but the morphological picture as yet gives no basis for distinguishing two kinds of microtubule.

An alternative interpretation for the presence of a doublet is possible. Mitotic apparatus microtubule protein might serve as a precursor for ciliary microtubules (65), perhaps after appropriate post-translational conversion. Possibly, the presence of two proteins has no functional significance in mitotic apparatus, but anticipates the conversion of the protein to ciliary protein.

Our results on the extraction of isolated mitotic apparatus can particularly be compared with those of Zimmerman (21) and Sakai (23). Zimmerman also extracted isolated mitotic apparatus with mercurial, but used an alkaline pH at which mitotic apparatus disperses. At this pH mitotic apparatus disperses also in the absence of mercurial, though considerably more slowly (our data). On analytical ultracentrifugation in 0.01 M mersalyl at pH 9, Zimmerman's preparation showed two boundaries, sedimenting with an S_{20} of 3.7 and 8.6, respectively.

Clearly, our protein should also be present in Zimmerman's preparation. Judging solely from sedimentation coefficients, either or both boundaries in Zimmerman's preparation could correspond to our protein. The 8.6S boundary could correspond to the 8.9S form of our protein. We do not obtain this sedimentation coefficient after dialysis at pH 9.0, but the 8.6S boundary also disappears from Zimmerman's preparations on dialysis. Zimmerman's 3.7S boundary could correspond to the 4.9S boundary we obtain, provided that the sedimentation coefficient of the protein is appropriately concentration dependent (cf. Stephens, reference 17).

Sakai (23) used a potassium chloride extraction, while also repeating Zimmerman's work. He described the potassium chloride extract as giving an ultracentrifuge pattern identical to that of Zimmerman's extract (except for the presence of 22S protein in the potassium chloride extract), but he assigned sedimentation coefficients of 3.5S and 13S to the two boundaries. The major, 3.5S, boundary in Sakai's preparation has been proposed to be microtubule protein (25). Sakai argues that the 3.5S and 13S boundaries represent the same protein, since both give way to a 2.5S boundary after sulfite reduction. However, there is a substantial discrepancy between the subunit molecular weight (34,000) reported for the 3.5S protein after sulfite reduction and the subunit molecular weight (52,000, more typical for microtubule protein) we obtain after reduction of our protein with mercaptoethanol in the presence of sodium dodecyl sulfate. To assure that the discrepancy was not due to a difference in the conditions of reduction, we dialyzed our protein overnight against a sulfite-containing medium identical to that employed by Sakai, and then proceeded to treatment with mercaptoethanol and sodium dodecyl sulfate. This caused no change in the estimated molecular weight. The exact relationship between our results and these previous ones is therefore not yet clear. However this may be resolved, it seems justifiable to assert that microtubule protein of mitotic apparatus is a typical microtubule protein.

We are grateful to Dr. J. H. Hash for performing the amino acid analysis. Mrs. Willenor Eaton gave skilled technical assistance throughout the work.

This work was supported by grant Nos. GB-7221 and GB-17741 from the National Science Foundation.

Received for publication 2 June 1970, and in revised form 13 July 1970.

Note Added in Proof: More recent studies (G. B. Witman. 1970. *J. Cell Biol.* **47** [2, Pt. 2]: 229 a.) have disputed the evidence that the two tubulins from outer doublets of flagella derrive from the *a*-tubule and *b*-tubule, respectively. This puts in question the analogy on the basis of which we suggested that a similar situation might exist in mitotic apparatus.

REFERENCES

1. TAYLOR, E. W. 1956. *J. Cell Biol.* **25**:145.
2. BORISY, G. G., and E. W. TAYLOR. 1967. *J. Cell Biol.* **34**:525.
3. SHELANSKI, M. L., and E. W. TAYLOR. 1967. *J. Cell Biol.* **34**:549.
4. ADELMAN, M. R., G. G. BORISY, M. L. SHELANSKI, R. C. WEISENBERG, and E. W. TAYLOR. 1968. *Fed. Proc.* **27**:1186.
5. SHELANSKI, M. L., and E. W. TAYLOR. 1968. *J. Cell Biol.* **38**:304.
6. WEISENBERG, R. C., G. G. BORISY, and E. W. TAYLOR. 1968. *Biochemistry.* **7**:4466.
7. DUTTON, G. R., and S. BARONDES. 1969. *Science (Washington).* **166**:1637.
8. MARANTZ, R. M., M. VENTILLA, and M. L. SHELANSKI. 1969. *Science (Washington).* **165**:498.
9. OLMSTED, J., K. CARLSON, R. KLEBE, F. RUDDLE, and J. ROSENBAUM. 1970. *Proc. Nat. Acad. Sci. U.S.A.* **65**:129.
10. PUSZKIN, S., and S. BERL. 1970. Nature (*London*). **225**:558.
11. BENSCH, K. G., and S. MALAWISTA. 1969. *J. Cell Biol.* **40**:95.
12. BENSCH, K. G., R. MARANTZ, H. WISNIEWSKI, and M. L. SHELANSKI. 1969. *Science (Washington).* **165**:495.
13. MARANTZ, R., and M. L. SHELANSKI. 1970. *J. Cell Biol.* **44**:234.
14. STEPHENS, R. E., F. L. RENAUD, and I. R. GIBBONS. 1967. *Science (Washington).* **156**:1606.
15. MOHRI, H. 1968. *Nature (London).* **217**:1053.
16. RENAUD, F. L., A. J. ROWE, and I. R. GIBBONS. 1968. *J. Cell Biol.* **36**:79.
17. STEPHENS, R. E. 1968. *J. Mol. Biol.* **32**:277.
18. STEPHENS, R. E. 1968. *J. Mol. Biol.* **33**:517.
19. STEPHENS, R. E. 1969. *Quart. Rev. Biophys.* **1**:377.
20. MAZIA, D., and K. DAN. 1952. *Proc. Nat. Acad. Sci. U.S.A.* **38**:826.
21. ZIMMERMAN, A. M. 1960. *Exp. Cell Res.* **20**:529.
22. DIRKSEN, E. R. 1964. *Exp. Cell Res.* **36**:256.
23. SAKAI, H. 1966. *Biochim. Biophys. Acta.* **112**:132.
24. KANE, R. E. 1967. *J. Cell Biol.* **32**:243.
25. KIEFER, B., H. SAKAI, A. J. SOLARI, and D. MAZIA. 1966. *J. Mol. Biol.* **20**:75.
26. BORISY, G. G., and E. W. TAYLOR. 1967. *J. Cell Biol.* **34**:535.
27. BIBRING, T., and J. BAXANDALL. 1968. *Science (Washington).* **161**:377.
28. MIKI-NOUMURA, T. 1968. *Exp. Cell Res.* **50**:54.
29. WEISENBERG, R. C., and E. W. TAYLOR. 1968. *Exp. Cell Res.* **53**:372.
30. STEPHENS, R. E. 1967. *J. Cell Biol.* **32**:255.
31. BIBRING, T., and J. BAXANDALL. 1969. *J. Cell Biol.* **41**:577.
32. STEPHENS, R. E., and R. W. LINCK. 1969. *J. Mol. Biol.* **40**:497.
33. KANE, R. E., and A. FORER. 1965. *J. Cell Biol.* **25**(3, Pt. 2):31.
34. FORER, A., and R. D. GOLDMAN. 1969. *Nature (London).* **222**:689.
35. GOLDMAN, R. D., and L. I. REBHUN. 1969. *J. Cell Sci.* **4**:179.
36. JACOBS, M., J. HOPKINS, and J. RANDALL. 1968. *J. Cell Biol.* **39**:66 a.
37. STEPHENS, R. E. 1970. *J. Mol. Biol.* **47**:353.
38. KANE, R. E. 1965. *J. Cell Biol.* **25**(1, Pt. 2):137.
39. HARRIS, P. 1962. *J. Cell Biol.* **14**:475.
40. LUFT, J. H. 1961. *J. Biophys. Biochem. Cytol.* **9**:409.
41. VENABLE, J. H., and R. COGGESHALL. 1965. *J. Cell Biol.* **25**:407.
42. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**:265.
43. LAYNE, E. 1957. *In* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press Inc., New York. **3**:450.
44. TAKAYAMA, K., D. H. MACLENNA., A. TZAGOLOF, and C. D. STONER. 1966. *Arch. Biochem.* **114**: 223.
45. ZAHLER, W., A. SAITO, and S. FLEISCHER. 1968. *Biochem. Biophys. Res. Commun.* **32**:512.
46. DAVIS, B. J. 1964. *Ann. N. Y. Acad. Sci.* **121**:404.
47. ORNSTEIN, L., and B. J. DAVIS. 1964. *Ann. N. Y. Acad. Sci.* **121**:321.
48. SHAPIRO, A. L., E. VIÑUELA, and J. V. MAIZEL. 1967. *Biochem. Biophys. Res. Commun.* **28**:815.
49. WEBER, K., and M. OSBORN. 1969. *J. Biol. Chem.* **244**:4406.
50. LINDBERG, U. 1967. *Biochemistry.* **6**:335.
51. OUCHTERLONY, Ö. 1949. *Ark. Kemi.* **26 B**:1.
52. MOORE, S., and W. H. STEIN. 1963. *In* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press Inc., New York. **6**:819.
53. SPACKMAN, D. H., W. H. STEIN, and S. MOORE. 1958. *Anal. Chem.* **30**:1190.
54. MOORE, S. 1968. *J. Biol. Chem.* **243**:6281.
55. RENAUD, F. L., A. J. ROWE, and I. R. GIBBONS. 1966. *J. Cell Biol.* **31**:92 A.

56. DUNKER, A. K., and R. R. RUECKERT. 1969. *J. Biol. Chem.* **244**:5074.
57. FULTON, C., R. E. KANE, and R. E. STEPHENS. 1971. *J. Cell Biol.* In press.
58. BIBRING, T., and G. H. COUSINEAU. 1964. *Nature (London).* **204**:805.
59. COHEN, W. D., and L. I. REBHUN. 1969. *J. Cell Sci.* **6**:159.
60. GIBBONS, I. R. 1965. *Arch. Biol.* **76**:317.
61. GIBBONS, I. R., and A. J. ROWE. 1965. *Science (Washington).* **149**:424.
62. GIBBONS, I. R. 1966. *J. Biol. Chem.* **241**:5599.
63. WILSON, H. J. 1969. *J. Cell Biol.* **40**:854.
64. HEPLER, P. K., J. R. MCINTOSH, and S. CLELAND. 1970. *J. Cell Biol.* **45**:438.
65. AUCLAIR, W., and B. W. SIEGEL. 1966. *Science (Washington).* **154**:915.