Sobue, K., Kanda, K., & Kakiuchi, S. (1982) *FEBS Lett. 150*, 185–190.
Steinert, P. M., Baliga, B. S., & Munro, H. N. (1974) *Anal. Biochem. 59*, 416–425.
Walters, B. B., & Matus, A. I. (1975) *Nature (London) 257*, 496–498.
Wang, Y.-J., & Mahler, H. R. (1976) *J. Cell Biol. 71*, 639–658.
Watterson, D. M., Sharief, F., & Vanaman, T. C. (1980) *J. Biol. Chem. 255*, 962–975.
Wood, J. G., Wallace, R. W., Whitaker, J. N., & Cheung, W. Y. (1980) *J. Cell Biol. 84*, 66–76.

Purification, Characterization, and Assembly Properties of Tubulin from Unfertilized Eggs of the Sea Urchin *Strongylocentrotus purpuratus*†

H. William Detrich, III,* and Leslie Wilson

ABSTRACT: Tubulin was purified from unfertilized eggs of the sea urchin *Strongylocentrotus purpuratus* by chromatography of an egg supernatant fraction on DEAE-Sephacel or DEAE-cellulose followed by cycles of temperature-dependent microtubule assembly and disassembly in vitro. After two assembly cycles, the microtubule protein consisted of the α- and β-tubulins (>98% of the protein) and trace quantities of seven proteins with molecular weights less than 55 000; no associated proteins with molecular weights greater than tubulin were observed. When analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on urea–polyacrylamide gradient gels, the α- and β-tubulins did not precisely comigrate with their counterparts from bovine brain. Two-dimensional electrophoresis revealed that urchin egg tubulin contained two major α-tubulins and a single major β species. No oligomeric structures were observed in tubulin preparations maintained at 0 °C. Purified egg tubulin assembled efficiently into microtubules when warmed to 37 °C in a glycerol-free polymerization buffer containing guanosine 5′-triphosphate. The critical concentration for assembly of once- or twice-cycled egg tubulin was 0.12–0.15 mg/mL. Morphologically normal microtubules were observed by electron microscopy, and these microtubules were depolymerized by exposure to low temperature or to podophyllotoxin. Chromatography of a twice-cycled egg tubulin preparation on phosphocellulose did not alter its protein composition and did not affect its subsequent assembly into microtubules. At concentrations above 0.5–0.6 mg/mL, a concentration-dependent "overshoot" in turbidity was observed during the assembly reaction. These results suggest that egg tubulin assembles into microtubules in the absence of the ring-shaped oligomers and microtubule-associated proteins that characterize microtubule protein from vertebrate brain.

The assembly and disassembly of labile cytoplasmic microtubules are apparently closely coupled with the functions that they perform. Because it is available in large quantity, most studies of cytoplasmic microtubule assembly have been performed on microtubule protein isolated from vertebrate brain tissue. However, it is important to recognize that brain microtubule protein may be an atypical material for study, perhaps modified to form stable cytoskeletal elements in nonmitotic cells. Therefore, we have chosen to investigate the properties of tubulin isolated from unfertilized eggs of the purple sea urchin, *Strongylocentrotus purpuratus*.

Unfertilized sea urchin eggs contain a large pool of presynthesized tubulin (Raff & Kaumeyer, 1973; Pfeffer et al., 1976). Bibring & Baxandall (1977) have shown that most of the tubulin incorporated into the mitotic spindle during the first cleavage division in *S. purpuratus* zygotes is drawn from this pool. Doublet-specific ciliary tubulin destined for ciliogenesis during the blastula stage is also present in the unfertilized egg (Bibring & Baxandall, 1981). However, the pool of ciliary tubulin in embryos appears to be small (capable of supporting three to four rounds of ciliary regeneration; Auclair & Siegel, 1966). Furthermore, Stephens (1978) was unable to detect ciliary tubulin in the unfertilized egg by peptide mapping. These studies suggest that the majority of the tubulin in the unfertilized egg is mitotic or cytoplasmic in destiny.

Dramatic changes both in the extent of assembly of cytoplasmic microtubules and in their organization take place during the first cleavage cycle of sea urchin zygotes. Few, if any, microtubules are assembled prior to fertilization in eggs of *Arbacia punctulata* and *Lytechinus variegatus* (Bestor & Schatten, 1981). Following fertilization in *S. purpuratus*, a monaster of microtubules forms around the sperm pronucleus and then recedes, to be replaced by the interphase asters and a spiral cortical array of microtubules (Harris et al., 1980a,b). The cortical microtubules and the interphase asters in turn break down prior to the formation of the mitotic spindle and the occurrence of the first cleavage at 135 min (15 °C). Harris et al. (1980b) have proposed that the breakdown of microtubules is induced by a transient, wavelike movement of some depolymerizing factor (perhaps calcium) from the cell center to the periphery of the cell.

Our long-range objective is to understand the mechanism and, ultimately, the regulation of the assembly and disassembly of sea urchin cytoplasmic microtubules, both in vitro and in vivo. In this report, we describe a protocol for the isolation of highly purified tubulin from unfertilized eggs of *S. purpuratus*. Furthermore, we have analyzed the protein com-

† From the Department of Biological Sciences, University of California, Santa Barbara, California 93106. *Received September 21, 1982; revised manuscript received January 13, 1983.* This work was supported by research grants from the U.S. Public Health Service (NS13560) and from the American Cancer Society (CD-3G) to L.W. H.W.D. was a Fellow in Cancer Research supported by Grant DRG 310-F of the Damon Runyon–Walter Winchell Cancer Fund (1979–1980); he was also supported by U.S. Public Health Service National Research Service Award GM07182 (1980–1982).

0006-2960/83/0422-2453$01.50/0

position and the assembly characteristics of this tubulin. The results suggest that study of this system will contribute significantly to our understanding of microtubule assembly. A preliminary report of some of this work has been published (Detrich et al., 1981).

Materials and Methods

Materials. 1,4-Piperazinediethanesulfonic acid (Pipes),[1] DTE, EGTA, GTP (type II-S), TAME, and Coomassie Brilliant Blue R-250 were obtained from Sigma Chemical Co. Coomassie Brilliant Blue G-250 was supplied by Polysciences. Sodium lauryl sulfate (specially pure) was purchased from BDH Chemicals, and ultrapure urea was produced by Schwarz/Mann. Ampholines were obtained from LKB and from Serva. All other chemicals were reagent grade.

DEAE-cellulose (Whatman DE-52) and DEAE-Sephacel (Pharmacia) were carefully equilibrated with the appropriate column buffer prior to use. Phosphocellulose (Whatman P-11) was precycled in 0.5 N HCl and 0.5 N NaOH according to the manufacturer's instructions. The precycled phosphocellulose was saturated with Mg^{2+} and equilibrated with the appropriate buffer as described by Williams & Detrich (1979).

Preparation of Unfertilized Eggs. Specimens of the purple urchin, *Strongylocentrotus purpuratus*, were collected locally in the Santa Barbara Channel and were maintained in tanks supplied with fresh, filtered seawater at 15–20 °C. Urchins were induced to spawn by injections of 1–2 mL of 0.55 M KCl into the body cavity, and ova were collected by inversion of female urchins over glass beakers containing filtered, natural seawater (15–20 °C). Following completion of spawning, eggs were collected by centrifugation (4000*g*, 4 °C, 2–4 min). The pelleted eggs were washed twice with filtered seawater: eggs were resuspended in 5 times (5×) their volume of seawater (15–20 °C), the suspension was stirred vigorously for 20–30 s to strip the jelly coats from the eggs, and dejellied eggs were collected by centrifugation as described previously. Finally, the eggs were washed once (by the procedure just described) with W buffer [0.1 M Pipes–NaOH, 2 mM EGTA, 1 mM $MgSO_4$, and 0.02% (w/v) NaN_3, pH 6.82], and the egg pellets were resuspended in an equal volume of PMI buffer [0.1 M Pipes–NaOH, 2 mM EGTA, 2 mM DTE, 1 mM $MgSO_4$, 1 mM TAME, 0.1 mM GTP, and 0.02% (w/v) NaN_3, pH 6.82].

Purification of Tubulin. Tubulin was purified from unfertilized eggs by a modification of the method of Kuriyama (1977). Suspensions of eggs in PMI buffer were homogenized in a Teflon–glass tissue homogenizer (four strokes, speed 9, Tri-R Stir-R Model S63C), and the homogenate was centrifuged at 210000*g* for 100 min at 4 °C. (All subsequent chromatographic operations were performed at 0–4 °C.) The lipid-containing layer at the top of each centrifuge tube was removed by aspiration, and the high-speed supernatants (HSS) were recovered and pooled. The HSS was either used immediately or prepared for storage by dropwise freezing in liquid nitrogen (see below). In pilot experiments (e.g., Figure 1), the HSS was applied to PMI-equilibrated columns of DEAE-cellulose or DEAE-Sephacel at a ratio of 1.5–2 mL of supernatant per 1 mL of exchanger. The columns were washed with 2–4 volumes of PMI buffer to recover nonbound proteins. Bound proteins were eluted by application of linear gradients of NaCl (0–0.5 M) in PMI buffer. Conductivities through the salt gradient were measured at room temperature with a Radiometer CDM 3 conductivity meter. After determination of the salt concentration required to elute tubulin from these exchangers, the chromatographic procedure was modified to permit batch adsorption and stepwise elution of tubulin. The HSS was adsorbed to the exchanger (2 mL of supernatant/1 mL of settled exchanger) for 30–45 min at 0–4 °C. The slurry was stirred occasionally to suspend the exchanger in the supernatant. Following adsorption, the exchanger was collected by centrifugation of the slurry (4000*g*, 2 min), and the nonbound supernatant was discarded. The resin was washed twice with 5× volumes of PMI buffer and then poured into an appropriate column. After the exchanger had packed, the column was developed with 2 or more volumes of PMI + 0.15 M NaCl to elute bound, nontubulin proteins. Tubulin was collected by application of PMI + 0.4 M NaCl to the column. Fractions were collected and assayed for protein (see below). Appropriate fractions were pooled, frozen dropwise in liquid nitrogen (Detrich & Williams, 1978), and then stored at −80 °C. When compared to the gradient elution protocol, this batch adsorption, step elution procedure facilitates the rapid processing of large volumes of HSS at the cost of slightly lower purity of the tubulin fractions obtained.

The partially purified tubulin obtained by DEAE ion-exchange chromatography (DEAE-tubulin) was further purified by cycles of temperature-dependent assembly and disassembly in vitro. Aliquots (10 mL) of the frozen DEAE-tubulin in PMI + 0.4 M NaCl were thawed rapidly and centrifuged (4000*g*, 10 min, 4 °C) to remove small amounts of denatured protein from the solution. The supernatant was desalted by passage over 1.5 × 20 cm columns of Bio-Gel P-2 equilibrated with PB buffer [0.1 M Pipes–NaOH, 1 mM EGTA, 1 mM $MgSO_4$, and 0.02% (w/v) NaN_3, pH 6.82]. GTP was added to the desalted DEAE-tubulin to a final concentration of 1 mM, and microtubules were assembled by warming the protein solution to 37 °C. After assembly was complete (as judged by turbidimetry; see below), the polymer was collected by centrifugation (40000*g*, 30 min, 35 °C). Microtubule pellets, H_1P [sample nomenclature is that of Borisy et al. (1975)], were resuspended in PB + 1 mM GTP (25–50% of the H_1S volume) to a final protein concentration of ca. 2 mg/mL, and the supernatants (H_1S) were discarded. Microtubules were depolymerized by incubation of the resuspended H_1P on ice for 30 min, and the depolymerized protein was clarified by centrifugation at 40000*g* for 30 min at 4 °C. The cold supernatant (C_1S) was recovered, while the cold pellet (C_1P) was discarded. An additional cycle of assembly and disassembly was performed to yield twice-cycled tubulin (C_2S). The C_2S was frozen dropwise in liquid nitrogen as described (Detrich & Williams, 1978) and stored at −80 °C.

Several additional comments on this method should be noted. First, the synthetic trypsin substrate TAME was included in PMI buffer to protect tubulin against proteolysis by cortical granule proteases that may be released during homogenization of the unfertilized eggs. Second, when large volumes (>20 mL) of DEAE-tubulin were purified further by assembly/disassembly selection, the protein was concentrated by ammonium sulfate fractionation. Ammonium sulfate was added gradually to the tubulin solution to a final concentration of 60% (w/v). After a 20-min incubation at 0 °C, the precipitate was collected by centrifugation (40000*g*, 15 min, 4 °C). The pellets were resuspended to 2–3 mg/mL in PB buffer, GTP was added to 1 mM, and the protein was as-

[1] Abbreviations: DEAE, diethylaminoethyl; DTE, dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine 5′-triphosphate; HSS, high-speed supernatant(s); MAP, microtubule-associated protein; Me_2SO, dimethyl sulfoxide; NaDodSO$_4$, sodium dodecyl (lauryl) sulfate; Pipes, 1,4-piperazinediethanesulfonic acid; PLN, podophyllotoxin; TAME, *p*-tosyl-L-arginine methyl ester hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

sembled without the desalting step described previously. The residual ammonium sulfate present in the resuspended protein had no effect on the subsequent assembly of the DEAE-tubulin. Third, in contrast to the method of Kuriyama (1977), the tubulin purified by this procedure has never been exposed to glycerol. Fourth, preparations of HSS, DEAE-tubulin, and C_2S have been stored as frozen beads at −80 °C for periods up to 6 months without loss of colchicine-binding activity or polymerization capacity.

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) on slab gels (15 × 15 × 0.15 cm) containing linear gradients of acrylamide (4–16%) and of urea (1–8 M) (Kim et al., 1979). Gels were fixed and stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in ethanol–acetic acid–water (45:10:45) for 8–12 h at room temperature and then destained with multiple changes of ethanol–acetic acid–water (25:10:65) over 2–3 days. All destained gels were stored in 7% (v/v) acetic acid. Gradient slab gels used for quantitative studies were fixed and stained with 1% Fast Green (Gorovsky et al., 1970) in 50% methanol–7% acetic acid (v/v) for 24 h (Berkowitz et al., 1977) and destained in 5% methanol–7% acetic acid (v/v). Appropriate lanes were cut out of the slabs and scanned at 640 nm with a Gilford Model 2400 spectrophotometer equipped with a linear transport device. The amount of protein in a given electrophoretic band was determined by integration of the corresponding peak in the resulting scan. Molecular weights of protein bands were estimated by comparison to protein standards (Pharmacia high and low molecular weight electrophoresis calibration kits) electrophoresed on the same gel (semilog plots of molecular weight vs. relative mobility).

Two-dimensional electrophoresis was performed according to the method of O'Farrell (1975) with some modifications. Isoelectric focusing on 4% polyacrylamide was performed in the first dimension on cylindrical gels (0.2 × 12 cm) containing 9.2 M urea, 2% (v/v) Triton X-100, 1% (w/v) Servalyt AG 5.0–5.5 (Serva), and 1% (w/v) ampholine, pH 4–6 (LKB), at room temperature. The gels were prerun as described (O'Farrell, 1975), the samples [in 9.5 M urea, 5% (v/v) 2-mercaptoethanol, and 2% (v/v) Triton X-100] were loaded onto the gels, and focusing was performed at 300 V for 20 h and then at 600 V for 1 h. Gels were removed, equilibrated for 1 h in $NaDodSO_4$ sample buffer [2% (w/v) $NaDodSO_4$, 5% (v/v) 2-mercaptoethanol, and 0.0625 M Tris-HCl, pH 6.8], and run in the second dimension on urea–polyacrylamide gradient slab gels (15 × 15 × 0.15 cm) according to the method of Laemmli (1970). Second-dimension slab gels were stained with Coomassie Brilliant Blue R-250 as described above. The pH gradient in the first dimension was determined by cutting duplicate gels into 5-mm sections, eluting the ampholytes from each section with 1 mL of water for 3 h, and measuring the pH of the solution at room temperature.

Polymerization Assays. Microtubule assembly was monitored turbidimetrically at 350 nm with a Gilford Model 2400 recording spectrophotometer equipped with a four-cell, thermostatable cuvette chamber. Polymerization reactions were performed in PB buffer supplemented with 1 mM GTP at 37 °C. Podophyllotoxin (PLN) was added when indicated from a 5 mM stock solution in Me_2SO.

Determination of Critical Concentrations. The critical concentration for assembly of the egg C_2S at 37 °C was determined by the quantitative sedimentation assay of Johnson & Borisy (1975). This procedure yields the equilibrium distribution of assembled microtubule protein in terms of two operationally defined species: polymer (microtubules) and the polymerizing unit (designated monomer). Briefly, samples (0.5 mL) of C_2S in PB + 1 mM GTP were incubated for 60 min at 37 °C to assemble microtubules. The polymerized samples were centrifuged at 28000*g* for 20 min at 37 °C, and the supernatants were recovered to yield the monomer fraction. The pellets were resuspended to the original sample volume in PB + 1 mM GTP, incubated at 0 °C for 30 min, and then centrifuged at 4 °C (28000*g*, 20 min) to remove irreversibly aggregated material. Finally, the cold supernatants were recovered to give the cold-reversible polymer fraction. Protein concentrations of the monomer and polymer fractions were determined as described below. Concentrations of protein in the monomer and cold-reversible polymer fractions were plotted as a function of the total C_2S concentration. The critical concentration was estimated as the *y* intercept of the linear regression line through those monomer (supernatant) data points obtained at total protein concentrations ≥0.15 mg/mL. Sedimentation of protein was specifically dependent upon assembly of tubulin, and polymer was quantitatively sedimented, on the basis of the criteria of Johnson & Borisy (1975): (1) microtubules were the predominant product of assembly under these conditions as revealed by negative-stain electron microscopy; (2) greater than 95% of the protein remained in the supernatant following incubation of the C_2S in the presence of 10^{-4} M colchicine; (3) approximately 90% of the protein in a microtubule pellet was recovered in the cold-reversible polymer fraction; and (4) the speed and duration of centrifugation produced a stable plateau in the mass of polymer sedimented.

The critical concentration for assembly of the C_2S (in PB + 1 mM GTP at 37 °C) was also evaluated by turbidimetry (Gaskin et al., 1974). Samples of C_2S at various initial concentrations were assembled to steady state (apparent equilibrium), and the change in absorbance at 350 nm was plotted vs. the total protein concentration. The apparent critical concentration was estimated by extrapolation of the linear regression line to the abscissa (zero turbidity, i.e., no assembly). Steady-state turbidity values corresponded to scattering by microtubule polymer as determined by negative-stain electron microscopy (cf. Figure 9); few nonmicrotubule aggregates were observed. Furthermore, increases in turbidity of the assembled C_2S were reversed to base-line levels by exposure of the samples to low temperature (0 °C). These observations indicate that the observed increases in turbidity were due to the reversible assembly of microtubules and exclude the presence of large amounts of irreversible aggregates.

Electron Microscopy. Microtubule samples for electron microscopy were diluted with warm PB + 1 mM GTP (37 °C) to a final protein concentration of 0.7–0.9 mg/mL. Following dilution, samples were immediately applied to carbon-coated collodion grids and negatively stained. One drop of sample was deposited on a grid for 30–60 s, excess solution was drawn off with filter paper, and the grid was rinsed with one to five drops of cytochrome *c* (1 mg/mL) and then with two to three drops of water. Excess water was drawn off with filter paper, and the samples were stained with several drops of 1% (w/v) aqueous uranyl acetate for 30–60 s. The last drop was removed with filter paper, and the grid was allowed to air-dry. Samples were observed with a Philips EM 300 electron microscope operated at 80 kV.

Samples of depolymerized solutions of microtubules were fixed for 5 min at 0 °C by 1:1 dilution with 2% aqueous glutaraldehyde. After the protein concentration was adjusted to 0.2–0.3 mg/mL with cold PB + 1 mM GTP (0 °C), sam-

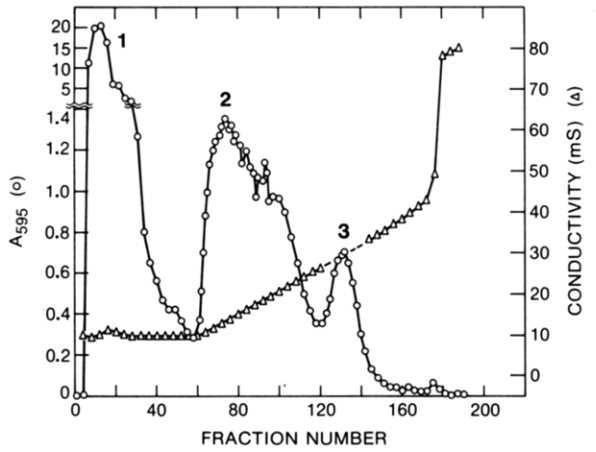

FIGURE 1: DEAE ion-exchange chromatography of the urchin egg HSS. The HSS (30 mL at 21 mg/mL in PMI) was adsorbed to a 1.6 × 10 cm column of DEAE-Sephacel equilibrated with the same buffer. The column was washed with PMI buffer (2 volumes) and then was developed with a 300-mL linear gradient from 0 to 0.5 M NaCl in PMI buffer (connected at fraction 48). At fraction 170, the column was eluted with PMI + 1 M NaCl. Fractions of 2.5 mL were collected. Protein was monitored by the method of Bradford (1976) and is plotted as the absorbance at 595 nm (O). Conductivities of the fractions (Δ) were measured at room temperature. Three peaks were observed and are numbered for reference in the text.

ples were applied to grids at room temperature, stained with uranyl acetate, and then observed as described above.

Miscellaneous Methods. Samples of urchin egg C_2S were chromatographed on columns of Mg^{2+}-saturated phosphocellulose equilibrated with PB + 0.1 mM GTP as described by Williams & Detrich (1979). The protein was applied at a ratio of 0.5 mg of C_2S/mL of bed volume, and tubulin, which does not bind to phosphocellulose under these conditions, was eluted with PB + 0.1 mM GTP. Finally, the column was washed with PB + 0.1 mM GTP + 0.8 M NaCl to release bound proteins. Bovine brain microtubule protein (C_3S) was purified through three cycles of assembly and disassembly in the absence of glycerol by the method of Asnes & Wilson (1979). Brain tubulin was separated from the microtubule-associated proteins (MAPs) by chromatography of the brain C_3S preparations on phosphocellulose (Detrich & Williams, 1978; Williams & Detrich, 1979). Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin as the standard.

Results

Purification of Tubulin from Unfertilized Eggs. Unfertilized eggs of the sea urchin *Strongylocentrotus purpuratus* contain a substantial pool of cytoplasmic tubulin (Pfeffer et al., 1976). We have purified this protein by DEAE ion-exchange chromatography followed by cycles of microtubule assembly and disassembly in vitro [cf. Kuriyama (1977)]. Figure 1 presents the elution profile obtained when the high-speed supernatant from unfertilized eggs (HSS) was chromatographed on a column of DEAE-Sephacel. Most of the protein did not bind to the ion-exchange resin under these conditions and eluted during the wash with PMI buffer (peak 1). Upon application of a linear gradient of NaCl (0–0.5 M) in PMI buffer at fraction 48, two additional, well-resolved peaks of protein were released. Finally, no additional protein was eluted when the column was washed with PMI + 1 M NaCl at fraction 170.

The protein compositions of the peaks were analyzed by electrophoresis in the presence of $NaDodSO_4$ on urea–polyacrylamide gradient gels (Figure 2). Figure 2A presents the

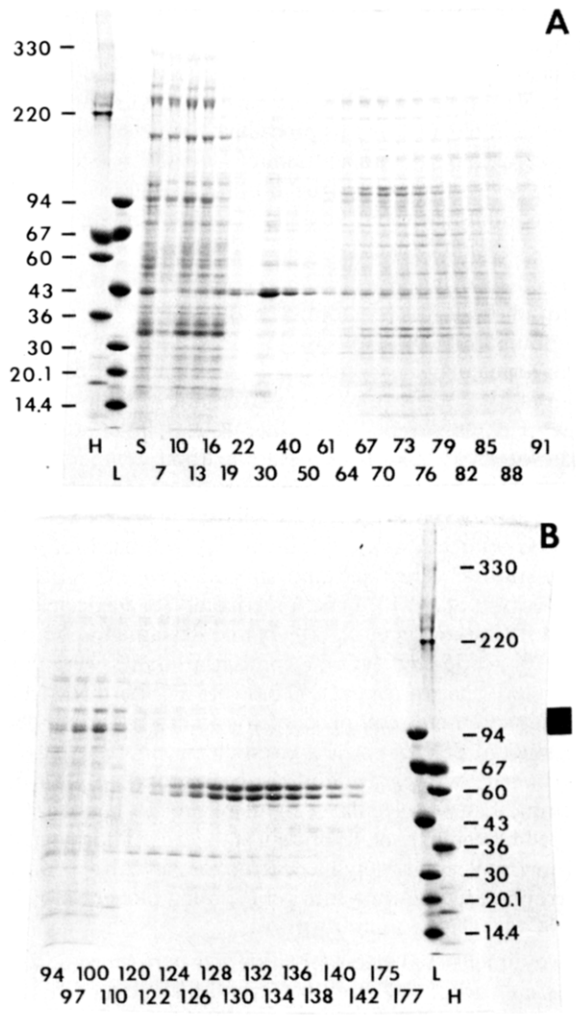

FIGURE 2: Electrophoretic analysis of protein fractions from the DEAE-Sephacel column of Figure 1. Aliquots of the indicated fractions were electrophoresed on $NaDodSO_4$–urea–polyacrylamide gradient gels (top to bottom = 4–16% acrylamide, 1–8 M urea) according to the method of Laemmli (1970) as modified by Kim et al. (1979). Gels were stained with Coomassie Brilliant Blue R-250 as described under Materials and Methods. Electrophoretic migration was from top to bottom. The molecular weights of standards are given in thousands on the vertical axis. Abbreviations: H, high molecular weight standards, including thyroglobulin (330 000), ferritin (220 000 half-unit), albumin (67 000), catalase (60 000), and lactate dehydrogenase (36 000); L, low molecular weight standards, including phosphorylase *b* (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100), and α-lactalbumin (14 400); S, high-speed supernatant (HSS). (A) Fractions from the nonbound peak 1 (7–50) and from peak 2 (61–91). The protein loads represent 1.5 μL of fractions S and 7–22 and 12.5 μL of fractions 30–91. (B) Fractions from the trailing edge of peak 2 (94–110), from peak 3 (120–142), and from the final high-salt wash (175–177). Protein loads represent 12.5 μL of each fraction.

protein compositions of fractions from the nonbound peak 1 (7–50) and from peak 2 (61–91), while Figure 2B shows the protein compositions of fractions from the trailing edge of peak 2 (94–110), from peak 3 (120–142), and from the final high-salt wash of the column (175–177). It is apparent that proteins corresponding to the α- and β-tubulins (apparent molecular weights of 60 700 ± 1300 and 56 100 ● 1100, respectively, on this gel system) were partly, if not entirely, removed from the HSS (gel lane S) following adsorption of the HSS to DEAE-Sephacel (compare lane S, Figure 2A, with the nonbound fractions 7–50 of peak 1). The nonadsorbed proteins of molecular weight 50 000–65 000 did not exactly comigrate with the tubulin bands, and, therefore, they probably

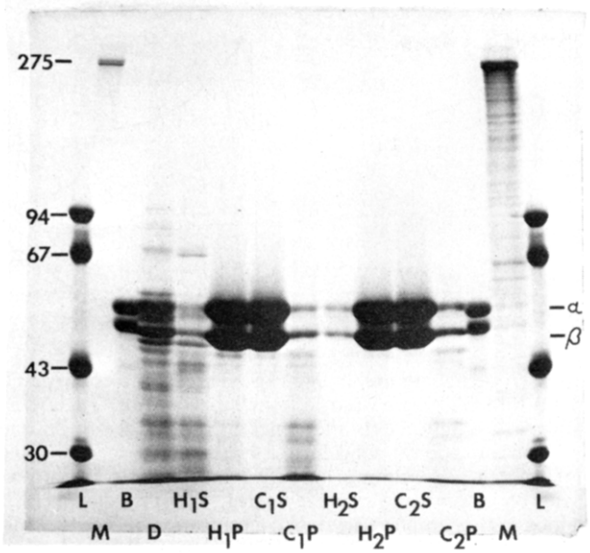

FIGURE 3: Electrophoretic analysis of fractions from two cycles of assembly and disassembly of DEAE ion-exchange-purified egg tubulin. Aliquots of the indicated fractions were electrophoresed on urea–polyacrylamide gradient gels as described in Figure 2 and under Materials and Methods. The molecular weights of standards are given in thousands on the vertical axis, and the approximate positions of the α- and β-tubulins are noted. Other details are given in the legend to Figure 2. Abbreviations: L, low molecular weight standards; M, bovine brain MAP 2; B, bovine brain, phosphocellulose-purified tubulin; D, desalted DEAE-tubulin; H_xS, H_xP, C_xS, C_xP, purification fractions obtained after x cycles of assembly/disassembly. "H" denotes fractions from 35 °C centrifugations, "C" denotes fractions from 4 °C centrifugations, "S" denotes supernatant, and "P" denotes pellet. This nomenclature is that of Borisy et al. (1975).

represent nontubulin components of the HSS. Peak 2 (fractions 61–110, Figure 2A,B) consisted of a wide spectrum of proteins released early in the salt gradient. Two major protein bands were present in the peak 3 fractions (120–142, Figure 2B). These bands may be identified as the α and β chains of the tubulin heterodimer on the basis of several criteria: (1) they are present in equimolar quantities and have the appropriate molecular weights and isoelectric points (see below); (2) they possess high affinity for DEAE-Sephacel as expected for the acidic tubulin chains; (3) peak 3 binds approximately 1 mol of colchicine per 110 000 g of protein (not shown); (4) the more slowly migrating chain of the doublet reacts with monoclonal antibodies to *S. purpuratus* flagellar α-tubulin (Asai et al., 1982); and (5) solutions of these proteins assemble microtubules when warmed to 37 °C in the presence of GTP (see below).

After establishing the salt concentrations necessary to elute tubulin from the DEAE ion exchangers, we modified the chromatographic procedure to permit batch adsorption and step elution of tubulin (see Materials and Methods). Electrophoretic analysis revealed that the partially purified tubulin obtained by this procedure (Figure 3, lane D) was slightly less pure than DEAE-tubulin isolated by gradient chromatography (cf. lanes 120–142, Figure 2B). Most of the additional contaminants had molecular weights less than those of the tubulin chains. This batch adsorption, step elution paradigm permits the rapid isolation of tubulin from large volumes of HSS, thereby minimizing denaturation of the labile tubulin chains.

DEAE-tubulin was further purified by cycles of temperature-dependent assembly and disassembly in vitro. Fractions from two assembly cycles were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as shown in Figure 3. Twice-cycled tubulin (C_2S) consisted of the α- and β-tubulins and *trace* amounts of three proteins with molecular weights

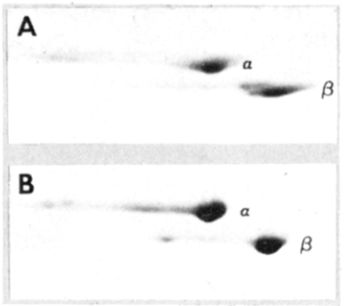

FIGURE 4: Two-dimensional electrophoretic analysis of bovine brain microtubule protein (C_3S) and of urchin egg tubulin (C_3S). Electrophoresis was performed according to the method of O'Farrell (1975) as described under Materials and Methods. Gels are oriented with the isoelectric focusing dimension on the horizontal axis (basic end on the left) and the molecular weight dimension on the vertical axis. The α- and β-tubulin regions are indicated. (A) Bovine brain C_3S. Only the tubulin region (ca. 11 μg of protein) is shown. (B) Urchin egg C_3S (15 μg).

of 41 000, 34 500, and 32 700. On other gradient gels and at higher protein loads, the material migrating at the dye front of the C_2S sample in Figure 3 was resolved into four additional proteins with molecular weights of 32 000, 30 500, 19 500, and 18 000. Tubulin was progressively enriched in the H_xP and C_xS fractions, while contaminants were recovered in the H_xS and C_xP samples. No proteins of molecular weight greater than the tubulin chains have been observed in C_1S or C_2S preparations. Finally, when analyzed at equivalent loadings on this gradient gel system, the α- and β-tubulins from sea urchin eggs did not precisely comigrate with their counterparts from bovine brain (data not shown). The α-tubulin from sea urchin eggs migrated more slowly than brain α-tubulin, while the converse was true for the β-tubulins from these two sources.

The purity of six different C_2S preparations was determined by quantitative densitometry of gradient slab gels stained with Fast Green. The results indicated that the α- and β-tubulins comprise greater than 98% of the C_2S. The remainder of the C_2S protein was distributed among the seven trace, low molecular weight polypeptides detected by Coomassie Blue staining.

We have examined the yield of tubulin obtained by these procedures. Between 30 and 60% of the total soluble pool of tubulin present in the unfertilized egg is recovered by DEAE ion-exchange chromatography. Following two cycles of assembly and disassembly in vitro, the C_2S represents 8–23% of the soluble tubulin pool. Proteolysis may contribute to the variability in the recovery of tubulin from the HSS by DEAE ion-exchange chromatography. The cortical granules of unfertilized sea urchin eggs contain proteases with properties similar to those of trypsin (Carroll & Epel, 1975; Fodor et al., 1975). Thus, disruption of cortical granules with release of their proteases during preparation of the HSS may be responsible for the variable recovery observed. We have noted that tubulin (monitored electrophoretically) is rapidly lost in HSS preparations lacking synthetic tryptic protease substrates. However, we have not systematically investigated the degree of protection afforded by the concentration of TAME employed in our HSS preparations. Most of the tubulin lost during subsequent purification by cycles of assembly and disassembly was found in the H_1S.

Complexity of Urchin Egg and Bovine Brain Tubulins. Two-dimensional electrophoresis according to the method of O'Farrell (1975) was employed to determine the chemical complexity of urchin egg tubulin compared to that of tubulin from bovine brain. Two α-tubulins and a single β species were observed in an urchin egg C_3S preparation (Figure 4B). In

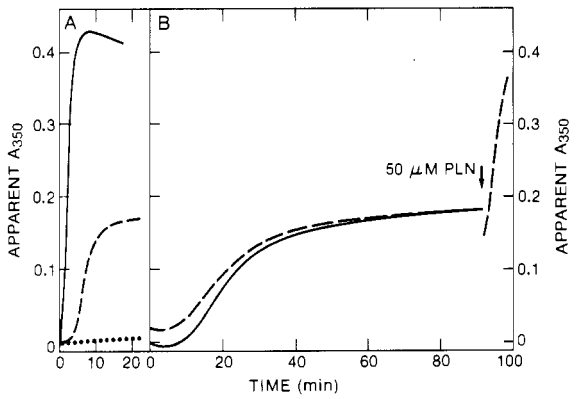

FIGURE 5: (A) Assembly of partially purified DEAE-tubulin. Three different tubulin samples in PB + 1 mM GTP were warmed from 0 to 37 °C at zero time, and assembly was monitored as the apparent absorbance at 350 nm. Protein concentrations: solid line, 2.4 mg/mL; dashed line, 1.2 mg/mL; dotted line, 0.59 mg/mL. (B) Reversible depolymerization of egg microtubules by low temperature. A sample of DEAE-tubulin (1.2 mg/mL) was assembled as described above (solid line). After 80 min, the sample was incubated on ice for 5 min and then assembled a second time (dashed line). Podophyllotoxin (PLN) was then added to a final concentration of 5×10^{-5} M at the point indicated by the arrow.

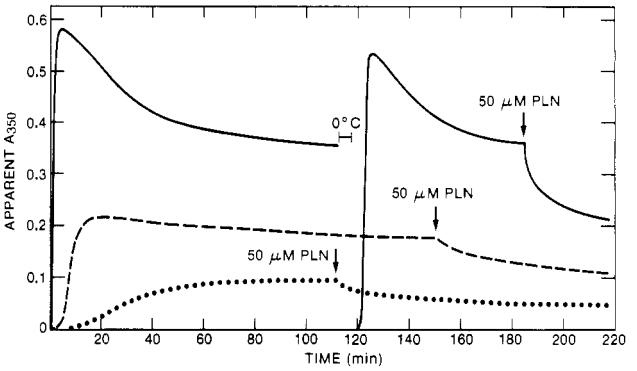

FIGURE 6: Assembly of urchin egg C_1S. Three samples of egg C_1S in PB + 1 mM GTP were warmed from 0 to 37 °C at zero time, and assembly was monitored as the apparent absorbance at 350 nm. Protein concentrations: solid line, 1.4 mg/mL; dashed line, 0.69 mg/mL; dotted line, 0.46 mg/mL. The sample at 1.4 mg/mL was incubated on ice for 5 min where indicated. Podophyllotoxin (PLN) was added to the samples to a final concentration of 5×10^{-5} M at the arrows.

contrast, two α-tubulins and four β-tubulins were detected in a brain C_3S preparation (Figure 4A). The lower complexity of egg tubulin (two major and two minor α's, one major and three minor β's) relative to bovine brain tubulin (up to 21 total α's and β's) has been confirmed by high-resolution isoelectric focusing studies (J. J. Correia, personal communication; H. P. Miller, H. W. Detrich, III, K. F. Sullivan, T. A. Pfeffer, and L. Wilson, unpublished experiments).

The isoelectric points (p*I*s) of the major urchin tubulin species were nearly identical with those of the brain α- and β-tubulins: the α's focused at a p*I* of approximately 5.5, whereas the p*I* of the β's was about 5.35. However, one of the sea urchin α-tubulins was slightly more acidic than the major brain α, and brain tubulin contained several minor β species that were more basic than the urchin β-tubulin. (The slight streaking of the tubulin bands toward the basic end of the isoelectric dimension appears to be due to overloading of the protein samples on the isoelectric gels.) Finally, the greater separation in the apparent molecular weight of the urchin α- and β-tubulins compared to that of the brain tubulins is clearly seen in these gels. In summary, these results suggest that there are chemical differences of unknown origin in the tubulins from unfertilized sea urchin eggs and from bovine brain.

Assembly Properties of Egg Tubulin. The assembly of DEAE-tubulin (in PB buffer + 1 mM GTP) at three different protein concentrations is shown in Figure 5A. Little assembly was observed at 0.59 mg/mL, whereas at 2.4 mg/mL an "overshoot" in turbidity was observed (see below). Normal assembly toward a steady-state plateau was observed at the intermediate protein concentration (1.2 mg/mL). The presence of microtubules in all three samples was verified by negative-stain electron microscopy. These microtubules were reversibly depolymerized by low temperatures: Figure 5B presents turbidity curves for two polymerizations of a single sample (1.2 mg/mL) of DEAE-tubulin. Following incubation on ice for 5 min, this sample repolymerized upon warming to 37 °C (dashed curve) to the level achieved during the first assembly (solid curve). It is noteworthy that the turbidity of this sample after the first assembly did not decrease to the previous base-line value upon incubation at 0 °C; this phenomenon has been observed with DEAE-tubulin with incubations at 0 °C for up to 30 min. The reason for this behavior is not understood, although it may be related to temperature-dependent denaturation of contaminating proteins, or possibly tubulin itself, in the DEAE preparation.

The assembly of one-cycled tubulin (C_1S) at three protein concentrations is presented in Figure 6. Several points can be made about these results. First, a concentration-dependent overshoot in turbidity was observed at concentrations above approximately 0.5–0.6 mg/mL: the turbidity increased rapidly to a peak upon warming and then decreased asymptotically toward a steady-state value. Second, the pattern of turbidity development and loss observed during a second assembly of the sample at highest concentration (solid curves, 1.4 mg/mL) was nearly identical with that observed during the first assembly. Third, the magnitude of the overshoot observed at a given tubulin concentration was not affected by the presence of a GTP-regenerating system (MacNeal et al., 1977) (data not shown). Fourth, all three samples depolymerized as expected following the addition of the antimitotic drug podophyllotoxin (Farrell et al., 1979a; Karr & Purich, 1979; Caplow & Zeeberg, 1981). In contrast, addition of podophyllotoxin to microtubules assembled from DEAE-tubulin led to a rapid increase in turbidity (Figure 5B). This phenomenon is probably related to the presence of the contaminating proteins in DEAE-tubulin.

Critical Concentration for Assembly of Egg Tubulin. Microtubule assembly systems in vitro are characterized by a critical concentration below which polymerization of microtubules does not occur (Gaskin et al., 1974; Borisy et al., 1975; Johnson & Borisy, 1975). Above this critical concentration, the extent of assembly is a linear function of the total protein concentration. We have employed a quantitative sedimentation assay (Johnson & Borisy, 1975) to determine the critical concentration for assembly of the egg C_2S at 37 °C. The equilibrium distribution of egg tubulin into monomer and polymer fractions (closed and open circles, respectively) as a function of the total protein concentration is shown in Figure 7. At low total C_2S concentrations (≤0.15 mg/mL), no polymer formed; all of the protein was recovered in the monomer fraction. However, above a critical concentration, the amount of assembled polymer was proportional to, and the concentration of monomer in equilibrium with polymer was nearly independent of, the total protein concentration. Extrapolation of the monomer line to the ordinate (neglecting the points at concentrations below 0.15 mg/mL where polymer

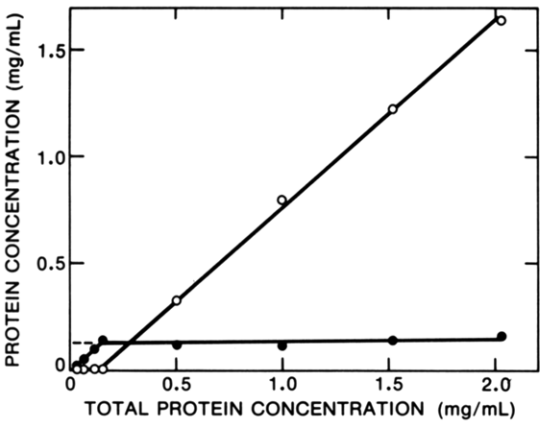

FIGURE 7: Concentration dependence of the monomer–polymer equilibrium at 37 °C. Samples of the urchin egg C_2S at various initial concentrations in PB + 1 mM GTP were incubated for 60 min at 37 °C to assemble microtubules. The polymerized samples were then resolved into monomer and cold-reversible polymer fractions as described under Materials and Methods. Concentrations of the monomer and cold-reversible polymer fractions (closed and open circles, respectively) for each sample were determined by the method of Bradford (1976) and are plotted as a function of the total C_2S concentration.

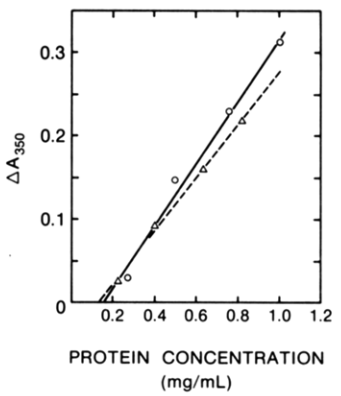

FIGURE 8: Turbidimetric determination of the critical concentration for assembly of urchin egg C_2S at 37 °C. Samples of egg C_2S in PB + 1 mM GTP were assembled to steady state as determined by turbidimetry (see Results). The change in absorbance at 350 nm (ΔA_{350}) is plotted vs. the protein concentration (○, solid line). A second aliquot of the same C_2S preparation was chromatographed on a column of Mg^{2+}-saturated phosphocellulose (see Materials and Methods), GTP was added to the tubulin fractions to a final concentration of 1 mM, and the samples were then assembled as above (△, dashed line).

did not form) gave a critical concentration for assembly of 0.12 mg/mL (dashed line). The slope of the monomer line indicated that the C_2S contained a small amount (1–2%) of inactive tubulin and/or contaminating, nonmicrotubule proteins. Thus, highly purified tubulin from sea urchin eggs is capable of self-assembly into microtubules at concentrations substantially below those required for assembly of pure brain tubulins (>2.5 mg/mL in the absence of glycerol or Me_2SO; Herzog & Weber, 1977; Himes et al., 1977). Although the identity of the monomer was not addressed in these experiments, Johnson & Borisy (1975, 1977) have demonstrated that the monomeric species present in equilibrium with brain microtubule polymer corresponds to the 6S tubulin dimer.

The critical concentration for assembly of the egg C_2S at 37 °C was also evaluated by turbidimetry (Gaskin et al., 1974). This analysis was complicated by the presence of the overshoot in turbidity at high C_2S concentration. However, electron microscopic studies revealed that microtubules were the predominant assembly product observed at steady state (i.e., following decay of the overshoot to apparent equilibrium; see Figure 9). Therefore, this method should produce a reliable *estimate* of the critical concentration of egg tubulin. Figure 8 presents a plot of steady-state turbidity vs. total C_2S concentration (circles). Extrapolation of the linear regression line to the abscissa (zero turbidity) gave an apparent critical concentration for assembly at 37 °C of 0.15 mg/mL. This value is similar to that obtained from the quantitative sedimentation analysis (0.12 mg/mL).

MAPs from brain tissue stimulate the assembly of microtubules from pure brain tubulin, thereby lowering the critical concentration for assembly. Both the high molecular weight MAPs (Sloboda et al., 1976) and τ proteins (Weingarten et al., 1975) may be separated from tubulin by chromatography of brain microtubule protein on columns of phosphocellulose. Prior chromatography of the egg C_2S on Mg^{2+}-saturated phosphocellulose (Williams & Detrich, 1979) had no effect on the critical concentration for assembly of this protein; the value obtained was 0.13 mg/mL (Figure 8, triangles). No change in the protein composition of the C_2S (i.e., no change in the numbers and amounts of low molecular weight proteins present with tubulin in the C_2S) was observed following phosphocellulose chromatography (not shown). Furthermore, no protein was recovered following application of PB buffer

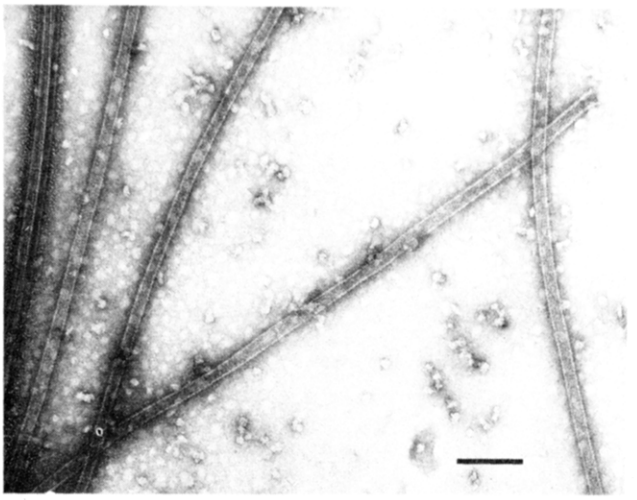

FIGURE 9: Electron micrograph of negatively stained microtubules assembled from egg tubulin (for preparation, see Materials and Methods). A C_1S preparation (2.7 mg/mL in PB + 1 mM GTP) was assembled at 37 °C, and a grid was prepared 71 min after the start of assembly. Bar = 0.1 μm.

containing 0.1 mM GTP and 0.8 M NaCl to the column (the ionic strength of this buffer is sufficient to release brain MAPs from phosphocellulose). Therefore, we suggest that the low critical concentration for assembly of the egg C_2S is a property of the tubulin itself and is not due to the presence of trace amounts of proteins with properties similar to those of the high molecular weight MAPs or τ proteins. However, we cannot exclude the possibility that the trace quantities of low molecular weight proteins present in the C_2S may influence the assembly of egg tubulin into microtubules.

Products of Assembly. We have examined the products of the assembly reaction by negative-stain electron microscopy. Prior to assembly, no oligomeric structures, such as rings, have been observed in solutions of egg tubulin maintained at 0–4 °C. However, when a C_1S preparation at high concentration (2.7 mg/mL) was assembled until the turbidity signal had decayed to near its apparent equilibrium value (71 min at 37 °C), only morphologically normal microtubules were observed (Figure 9). The rows of subunits called protofilaments are apparent in these microtubules. Furthermore, the microtubules tend to be very long (average length 10–20 μm), and they

associate laterally to a significant extent.

Discussion

Spontaneous assembly of microtubules in supernatants prepared from homogenates of sea urchin eggs is inhibited (Burns & Starling, 1974; Bryan, 1975; Kane, 1975), perhaps due to the presence of high concentrations of RNA (Bryan et al., 1975). Thus, an initial assembly-dependent purification of microtubule protein from sea urchin eggs is not possible as it is with brain systems (Shelanski et al., 1973). We have developed an alternative chromatographic procedure, based on that of Kuriyama (1977), for the initial, large-scale purification of tubulin from unfertilized eggs of *S. purpuratus*. The DEAE-tubulin may be further purified to near-homogeneity by cycles of temperature-dependent assembly and disassembly in vitro. Large quantities of highly purified egg tubulin can be rapidly isolated by these methods. Purified sea urchin egg tubulin is of relatively low complexity compared to brain tubulins, and it assembles efficiently into microtubules with a low critical concentration (at 37 °C) in the apparent absence of MAPs related to those of brain microtubule protein. Finally, the egg tubulin shows an unusual overshoot in turbidity during assembly at moderate protein concentration (>0.5–0.6 mg/mL).

Purification and Characterization of Egg Tubulin. Chromatography of a high-speed extract of sea urchin eggs on DEAE-Sephacel was employed to isolate a fraction enriched in two proteins with the properties of the α- and β-tubulins (see Results). The urchin egg tubulins migrate similarly to, but not identically with, the brain tubulins on $NaDodSO_4$–urea–polyacrylamide gradient gels (data not shown). In addition, both the α and β chains migrate with anomalously high molecular weights (60 700 and 56 100, respectively) on the urea–polyacrylamide gradient gel system employed here. This behavior may be the result of reduced binding of $NaDodSO_4$ by the tubulin chains in the presence of urea (Raff & Kaumeyer, 1973; Bibring et al., 1976; Clayton et al., 1980).

When DEAE-tubulin was subjected to two cycles of assembly/disassembly in vitro, the resulting C_2S contained the α- and β-tubulins (>98% of the total protein) and trace quantities of seven proteins with molecular weights less than 55 000. No associated proteins corresponding to the τ proteins (M_r 55 000–64 000; Weingarten et al., 1975; Cleveland et al., 1977a) or to the high molecular weight MAPs (M_r 275 000–325 000; Murphy & Borisy, 1975; Sloboda et al., 1975) of microtubule protein from vertebrate brain tissue have been observed in C_1S or C_2S preparations from urchin eggs. However, these proteins would not be expected to cofractionate with tubulin during DEAE ion-exchange chromatography (Murphy et al., 1977). The significance of the low molecular weight proteins is unclear. Some of them may be related to the low molecular weight MAPs (M_r 30 000–35 000) present in microtubule protein isolated from bovine brain (Berkowitz et al., 1977). Alternatively, they may represent microtubule-binding, assembly-promoting polypeptides similar to the fragments of MAP 2 (M_r 32 000–39 000) produced by proteolytic cleavage (Vallee, 1980). However, preliminary evidence suggests that some of the low molecular weight proteins may actually be breakdown products of tubulin produced when tubulin samples are boiled in $NaDodSO_4$ electrophoresis sample buffer (H. W. Detrich, III, and L. Wilson, unpublished experiments). [Cleavage of proteins upon boiling in the presence of $NaDodSO_4$ and 2-mercaptoethanol has also been reported by Kowit & Maloney (1982).] Thus, the actual purity of our C_2S preparations may be greater than that stated here.

Tubulins from a variety of vertebrate brain tissues display a high degree of heterogeneity (Gozes & Littauer, 1978; Marotta et al., 1978; Nelles & Bamburg, 1979). George et al. (1981) have demonstrated that calf brain tubulin can be resolved by isoelectric focusing into at least 17 chemically distinct species. With the lower isoelectric resolution provided by two-dimensional electrophoresis, we have observed six tubulin species in microtubule protein isolated from bovine brain (Figure 4A). In contrast, two α-tubulins and a single β-tubulin were observed in an urchin egg tubulin preparation (Figure 4B). It is possible that the lower complexity of egg tubulin contributes to its facile assembly into microtubules in the apparent absence of MAPs (see below).

Recently, Asai et al. (1982) have reported that monoclonal antibodies to urchin flagellar outer doublet α-tubulin reveal quantitative differences in immunoreactivity between bovine brain and urchin egg α-tubulins. Taken together, the differences in complexity and in immunoreactivity suggest that the tubulins from bovine brain and from sea urchin eggs differ chemically, presumably reflecting variations in the primary sequences of the tubulin chains and/or in the nature of any posttranslational modifications.

Assembly Properties of Egg Tubulin. Purified egg tubulin assembled readily into microtubules when C_1S or C_2S preparations were warmed in the presence of GTP (Figures 6–9). Morphologically normal microtubules were formed (Figure 9), and these microtubules were depolymerized by exposure to low temperature or to the drug podophyllotoxin (Figure 6). The apparent critical concentration for assembly at 37 °C, determined both by sedimentation analysis and by turbidimetric analysis, was 0.12–0.15 mg/mL (Figures 7 and 8). Chromatography of an egg C_2S preparation on Mg^{2+}-saturated phosphocellulose had no effect on its ability to assemble into microtubules (Figure 8), and the protein composition of the chromatographed tubulin was identical with that of control, unchromatographed material. Similar observations have been described in two preliminary reports by Suprenant and coworkers (Suprenant & Rebhun, 1980; Suprenant et al., 1981). They purified tubulin from unfertilized eggs of *S. purpuratus* by a different method (Kuriyama, 1977) and reported critical concentrations for its assembly of 0.2 mg/mL at 37 °C and 0.81 mg/mL at 15 °C. In contrast, the critical concentration for assembly of phosphocellulose-purified, MAP-free brain tubulin, >2.5 mg/mL in the absence of glycerol or Me_2SO (Herzog & Weber, 1977; Himes et al., 1977), is at least an order of magnitude greater than the critical concentration for assembly of whole brain microtubule protein (Gaskin et al., 1974). Therefore, we suggest that the capability for efficient self-assembly into microtubules is an intrinsic property of egg tubulin itself and is not due to the presence in these preparations of trace amounts of proteins with properties similar to those of the τ proteins or high molecular weight MAPs of brain microtubule protein. This conclusion is consistent with reports that tubulins purified from dogfish brain (Langford, 1978), from Ehrlich ascites tumor cells (Doenges et al., 1979), from yeast cells (Kilmartin, 1981), and from sperm outer doublet microtubules (Kuriyama, 1976; Binder & Rosenbaum, 1978; Farrell & Wilson, 1978), spindle microtubules (Keller & Rebhun, 1982), and egg cytoplasm (Kuriyama, 1977) of the sea urchin assemble into microtubules at low protein concentration in the apparent absence of MAPs. However, we cannot rule out the possibility that one or more of the low molecular weight proteins present in our purified egg tubulin preparations function as an assembly-promoting, associated protein.

The critical concentration for assembly of spindle tubulin from *S. purpuratus* embryos, 0.15–0.2 mg/mL at 37 °C in a buffer essentially identical with PB + 1 mM GTP (Keller & Rebhun, 1982), is similar to that reported here for tubulin isolated from unfertilized eggs. This suggests that spindle tubulin does not represent a specialized, chemically distinct subset of egg tubulin. Under similar conditions, pure tubulin derived from sperm outer doublet microtubules of *S. purpuratus* assembles into singlet microtubules with a critical concentration of 0.55–0.72 mg/mL at 35–37 °C (Binder & Rosenbaum, 1978; Farrell & Wilson, 1978; Farrell et al., 1979b). This disparity may reflect differences in the chemical structures of these functionally distinct tubulins from the same species.

We have examined the products of assembly of egg tubulin by negative-stain electron microscopy. Although both sheetlike structures and microtubules have been observed during the early stages of assembly of egg tubulin, microtubules were the predominant, if not exclusive, structure observed at turbidimetric steady state (Figure 9). We note that the critical concentration derived from the turbidity data (0.13–0.15 mg/mL; Figure 8) is nearly identical with that obtained by the quantitative sedimentation analysis (0.12 mg/mL; Figure 7). Furthermore, the turbidity generated by C_1S or C_2S preparations was completely reversible by exposure to low temperature. These results indicate that the turbidity observed at steady state corresponds to scattering by microtubules. We conclude that turbidimetric analysis can be employed to obtain reliable estimates for the critical concentration for assembly of egg tubulin.

MAPs from vertebrate brain tissue induce the formation of ring-shaped oligomers when added to purified brain tubulin at low temperature (Murphy & Borisy, 1975; Weingarten et al., 1975; Sloboda et al., 1976; Cleveland et al., 1977a,b). Several investigators have proposed that the rings of tubulin and MAPs function as obligate nucleation centers for microtubule assembly (Erickson, 1974; Kirschner et al., 1975). However, the direct participation of rings as nuclei has been challenged by the observation that rings beak down to smaller structures prior to the onset of microtubule assembly (Mandelkow et al., 1980). We have not observed rings in solutions of egg tubulin maintained at 0–4 °C, and formation of rings has not been detected during the assembly reaction (M. A. Jordan and H. W. Detrich, III, unpublished results). Furthermore, the egg C_2S exhibited a single, homogeneous boundary ($s_{20,w}$ = 5.2 S at 1.1 mg/mL) corresponding to the tubulin dimer when sedimented in PB buffer + 0.1 mM GTP at 4 °C; no evidence of higher oligomers of tubulin was observed (J. J. Correia, personal communication). Few, if any, rings are present in other MAP-free assembly systems (Kuriyama, 1977; Binder & Rosenbaum, 1978; Langford, 1978; Doenges et al., 1979). Therefore, these observations suggest that other structures formed directly from tubulin function as nuclei for microtubule assembly. Erickson & Pantaloni (1981) have proposed theoretical pathways for the nucleation of microtubule assembly through the formation of tubulin sheets. Clearly, further experiments are warranted to determine the mechanism of microtubule assembly from solutions of pure tubulin.

We have observed an unusual concentration-dependent overshoot in turbidity during assembly of egg tubulin at concentrations above 0.5–0.6 mg/mL at 37 °C (Figure 7). A similar turbidity overshoot has been observed during assembly at physiological temperature (18 °C) and concentration (>1.2 mg/mL) (H. W. Detrich, III, M. A. Jordan, R. C. Williams, Jr., and L. Wilson, unpublished experiments). Thus, the overshoot is not an artifact due solely to assembly at elevated temperature. The turbidity generated during microtubule assembly is thought to be proportional to the assembled mass and to be independent of the length of the microtubules formed for microtubules longer than approximately the wavelength of the incident light (Gaskin et al., 1974). Thus, one possible explanation for the overshoot is that more polymer is assembled initially than is stable at steady state or apparent equilibrium. The observation of turbidity overshoots in tubulin solutions containing a GTP-regenerating system rules out polymer instability due to rapid depletion of GTP during assembly. Alternatively, some physical process such as a rapid redistribution of microtubule lengths (toward very short microtubules) or slow conversion of sheets of protofilaments into microtubules may produce a decrease in the optical signal without affecting the mass of assembled polymer. We are currently investigating the mechanism underlying the turbidity overshoot in greater detail.

Most studies of microtubule assembly have been performed on microtubule protein obtained from vertebrate brain. Due to the variable and complex composition of this material (presence of rings of various morphologies, quantitative and qualitative differences in MAP composition), several conflicting models of microtubule assembly have been advanced [for a recent review, see Kirschner (1979)]. In contrast, the assembly of highly purified tubulin from sea urchin eggs proceeds efficiently in the apparent absence of both rings and MAPs. Thus, analysis of this assembly system may lead to new insights concerning the mechanism of microtubule assembly.

Added in Proof

A paper describing the purification and characterization of tubulin from *Strongylocentrotus purpuratus* eggs has recently been published (Suprenant & Rebhun, 1983).

Acknowledgments

We thank Dr. David J. Asai, Dr. John J. Correia, Dr. Mary Ann Jordan, Dr. Robley C. Williams, Jr., Vincent D. Lee, and Kevin F. Sullivan for many stimulating discussions and suggestions. We also acknowledge the excellent technical assistance rendered by Herbert P. Miller.

References

Asai, D. J., Brokaw, C. J., Thompson, W. C., & Wilson, L. (1982) *Cell Motil. 2*, 599–614.

Asnes, C. F., & Wilson, L. (1979) *Anal. Biochem. 98*, 64–73.

Auclair, W., & Siegel, B. W. (1966) *Science (Washington, D.C.) 154*, 913–915.

Berkowitz, S. A., Katagiri, J., Binder, H. K., & Williams, R. C., Jr. (1977) *Biochemistry 16*, 5610–5617.

Bestor, T. H., & Schatten, G. (1981) *Dev. Biol. 88*, 80–91.

Bibring, T., & Baxandall, J. (1977) *Dev. Biol. 55*, 191–195.

Bibring, T., & Baxandall, J. (1981) *Dev. Biol. 83*, 122–126.

Bibring, T., Baxandall, J., Denslow, S., & Walker, B. (1976) *J. Cell Biol. 69*, 301–312.

Binder, L. I., & Rosenbaum, J. L. (1978) *J. Cell Biol. 79*, 500–515.

Borisy, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B., & Johnson, K. A. (1975) *Ann. N.Y. Acad. Sci. 253*, 107–132.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bryan, J. (1975) *Am. Zool. 15*, 649–660.

Bryan, J., Nagle, B. W., & Doenges, K. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3570–3574.

Burns, R. G., & Starling, D. (1974) *J. Cell Sci. 14*, 411–419.
Caplow, M., & Zeeberg, B. (1981) *J. Biol. Chem. 256*, 5608–5611.
Carroll, E. J., Jr., & Epel, D. (1975) *Dev. Biol. 44*, 22–32.
Clayton, L., Quinlan, R. A., Roobol, A., Pogson, C. I., & Gull, K. (1980) *FEBS Lett. 115*, 301–305.
Cleveland, D. W., Hwo, S.-Y., & Kirschner, M. W. (1977a) *J. Mol. Biol. 116*, 207–225.
Cleveland, D. W., Hwo, S.-Y., & Kirschner, M. W. (1977b) *J. Mol. Biol. 116*, 227–247.
Detrich, H. W., III, & Williams, R. C., Jr. (1978) *Biochemistry 17*, 3900–3907.
Detrich, H. W., III, Miller, H. P., Snyder, K. B., Pfeffer, T. A., & Wilson, L. (1981) *J. Cell Biol. 91*, 330a.
Doenges, K. H., Weissinger, M., Fritzsche, R., & Schroeter, D. (1979) *Biochemistry 18*, 1698–1702.
Erickson, H. P. (1974) *J. Supramol. Struct. 2*, 393–411.
Erickson, H. P., & Pantaloni, D. (1981) *Biophys. J. 34*, 293–309.
Farrell, K. W., & Wilson, L. (1978) *J. Mol. Biol. 121*, 393–410.
Farrell, K. W., Kassis, J. A., & Wilson, L. (1979a) *Biochemistry 18*, 2642–2647.
Farrell, K. W., Morse, A., & Wilson, L. (1979b) *Biochemistry 18*, 905–911.
Fodor, E. J. B., Ako, H., & Walsh, K. A. (1975) *Biochemistry 14*, 4923–4927.
Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737–758.
George, H. J., Misra, L., Field, D. J., & Lee, J. C. (1981) *Biochemistry 20*, 2402–2409.
Gorovsky, M. L., Carlson, K., & Rosenbaum, J. L. (1970) *Anal. Biochem. 35*, 359–370.
Gozes, I., & Littauer, U. Z. (1978) *Nature (London) 276*, 411–413.
Harris, P., Osborn, M., & Weber, K. (1980a) *Exp. Cell Res. 126*, 227–236.
Harris, P., Osborn, M., & Weber, K. (1980b) *J. Cell Biol. 84*, 668–679.
Herzog, W., & Weber, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1860–1864.
Himes, R. H., Burton, P. R., & Gaito, J. M. (1977) *J. Biol. Chem. 252*, 6222–6228.
Johnson, K. A., & Borisy, G. G. (1975) in *Molecules and Cell Movement* (Inoué, S., & Stephens, R. E., Eds.) pp 119–139, Raven Press, New York.
Johnson, K. A., & Borisy, G. G. (1977) *J. Mol. Biol. 117*, 1–31.
Kane, R. G. (1975) *J. Cell Biol. 66*, 305–315.
Karr, T. L., & Purich, D. L. (1979) *J. Biol. Chem. 254*, 10885–10888.
Keller, T. C. S., III, & Rebhun, L. I. (1982) *J. Cell Biol. 93*, 788–796.
Kilmartin, J. V. (1981) *Biochemistry 20*, 3629–3633.
Kim, H., Binder, L. I., & Rosenbaum, J. L. (1979) *J. Cell Biol. 80*, 266–276.
Kirschner, M. W. (1979) *Int. Rev. Cytol. 54*, 1–71.
Kirschner, M. W., Honig, L. S., & Williams, R. C. (1975) *J. Mol. Biol. 99*, 263–276.
Kowit, J. D., & Maloney, J. (1982) *Anal. Biochem. 123*, 86–93.
Kuriyama, R. (1976) *J. Biochem. (Tokyo) 80*, 153–165.
Kuriyama, R. (1977) *J. Biochem. (Tokyo) 81*, 1115–1125.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Langford, G. M. (1978) *Exp. Cell Res. 111*, 139–151.
MacNeal, R. K., Webb, B. C., & Purich, D. L. (1977) *Biochem. Biophys. Res. Commun. 74*, 440–447.
Mandelkow, E.-M., Harmsen, A., Mandelkow, E., & Bordas, J. (1980) *Nature (London) 287*, 595–599.
Marotta, C. A., Harris, J. L., & Gilbert, J. M. (1978) *J. Neurochem. 30*, 1431–1440.
Murphy, D. B., & Borisy, G. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2696–2700.
Murphy, D. B., Vallee, R. B., & Borisy, G. G. (1977) *Biochemistry 16*, 2598–2605.
Nelles, L. P., & Bamburg, J. R. (1979) *J. Neurochem. 32*, 477–489.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Pfeffer, T. A., Asnes, C. F., & Wilson, L. (1976) *J. Cell Biol. 69*, 599–607.
Raff, R. A., & Kaumeyer, J. F. (1973) *Dev. Biol. 32*, 309–320.
Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.
Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L., & Greengard, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 177–181.
Sloboda, R. D., Dentler, W. L., & Rosenbaum, J. L. (1976) *Biochemistry 15*, 4497–4505.
Stephens, R. E. (1978) *Biochemistry 17*, 2882–2891.
Suprenant, K. A., & Rebhun, L. I. (1980) *J. Cell Biol. 87*, 246a.
Suprenant, K. A., & Rebhun, L. I. (1983) *J. Biol. Chem. 258*, 4518–4525.
Suprenant, K. A., Bender, P., & Rebhun, L. I. (1981) *J. Cell Biol. 91*, 328a.
Vallee, R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3206–3210.
Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858–1862.
Williams, R. C., Jr., & Detrich, H. W., III (1979) *Biochemistry 18*, 2499–2503.