Temperature and pH govern the self-assembly of microtubules from unfertilized sea-urchin egg extracts

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Summary

A new method for microtubule purification from unfertilized sea-urchin eggs was developed in order to obtain large quantities of calcium- and cold-labile microtubules that contained microtubule-associated components important for mitosis. By taking into consideration the pH, ionic composition of egg cytoplasm, and the physiological temperature for growth of the Pacific coast sea-urchin *Strongylocentrotus purpuratus*, methods were developed for the assembly of intact microtubules directly from unfertilized egg extracts. The microtubules obtained by cycles of temperature-dependent assembly and disassembly are composed of tubulin and abundant microtubule-associated proteins. These microtubules are cold- and calcium-labile and assemble at a critical protein concentration of 0·11 mg ml^{-1} at 24°C. The yield of microtubule protein obtained by this new method is equivalent to that obtained with taxol (6–8 mg/20 ml packed eggs). Microtubules that have been fixed and prepared for electron microscopy are decorated with large, globular projections that are attached to the microtubule by thin stalks.

Key words: microtubule-associated proteins, self-assembly, sea-urchin egg.

Introduction

Sea-urchin eggs are large cells that can be isolated in quantities suitable for biochemical analysis of mitosis. Fertilization triggers the synchronous development of large mitotic spindles that are easily visualized in the light microscope and isolated in large quantities (Salmon, 1982; Suprenant, 1986). In addition, the unfertilized egg contains a large pool of soluble tubulin (Pfeffer *et al.* 1976; Raff & Kaumeyer, 1973) and microtubule-associated proteins (MAPs) (Vallee & Bloom, 1983; Scholey *et al.* 1984). Because the unassembled pool of tubulin in the unfertilized egg is incorporated directly into the microtubules of the first division mitotic apparatus (Bibring & Baxandall, 1977), the unfertilized egg is an appropriate source of microtubule protein destined for mitosis.

Attempts to assemble microtubules directly from unfertilized egg extracts under conditions that support the assembly of vertebrate brain microtubules have failed (Kane, 1975), although a limited amount of assembly is obtained if short pieces of rat brain microtubules are added as 'seeds' (Burns & Starling, 1974). Authors from several laboratories have suggested that RNA or other polyanions (Bryan, 1975; Bryan *et al.* 1975), polysacharides (Naruse & Sakai, 1980), or proteases (Asnes & Wilson, 1981) prevent microtubule assembly in sea-urchin egg extracts although the effectiveness of these inhibitors *in vivo* is not known. Egg microtubules can be assembled *in vitro* if the tubulin is first purified by anion-exchange chromatography (Kuriyama, 1977); however, the chromatographic methods used yield essentially MAP-free microtubules (Suprenant & Rebhun, 1983; Detrich & Wilson, 1983). The anti-tumour drug, taxol, has been used to prepare MAP-containing microtubules from eggs (Vallee & Bloom, 1983; Scholey *et al.* 1984), but these microtubules are calcium- and cold-insensitive and are, therefore, inadequate preparations for studies of the dynamic aspects of microtubules, similar to those known to occur during mitosis.

A new method for the isolation of microtubule protein from sea-urchin eggs was developed in order

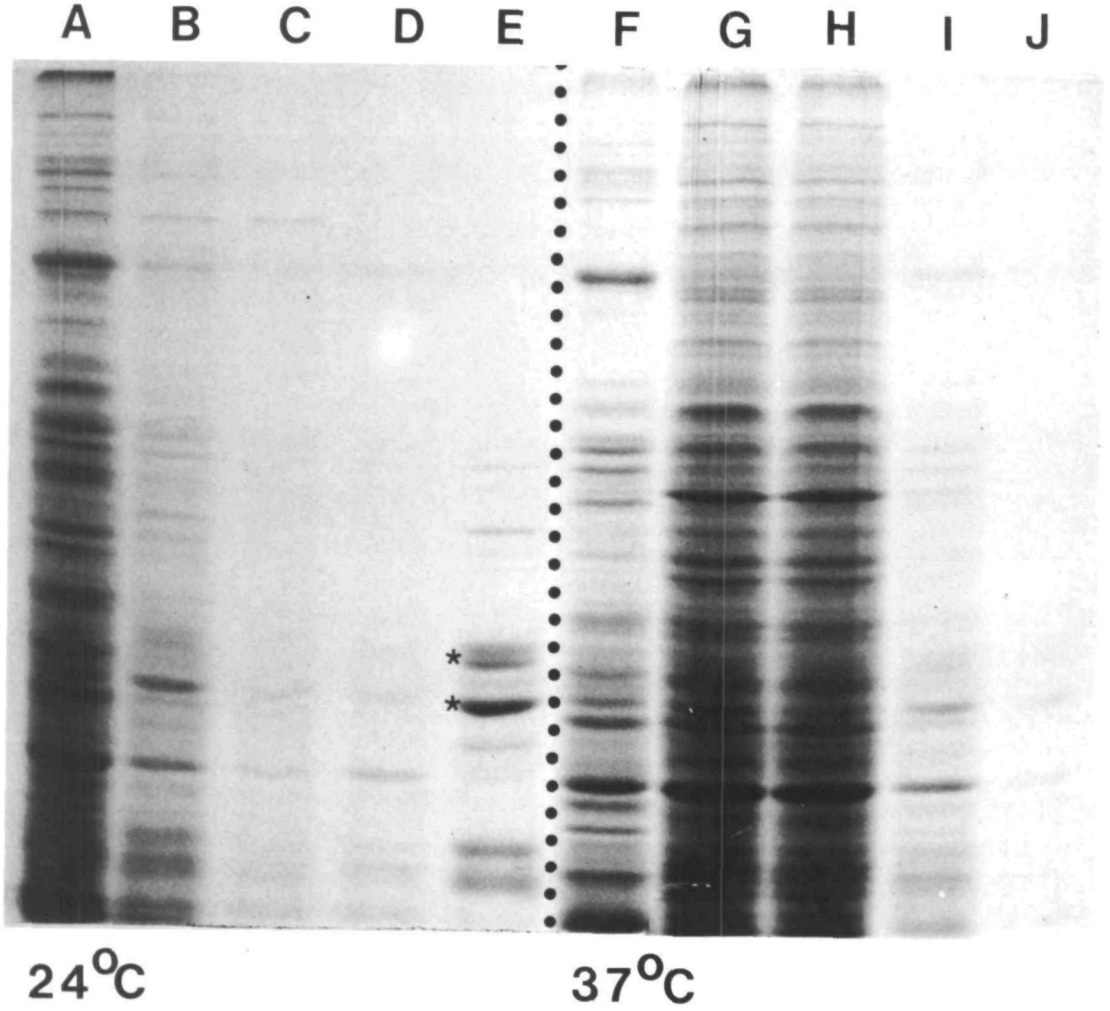

Fig. 1. Temperature effects on egg microtubule assembly in unfertilized sea-urchin egg extracts. Coomassie Blue-stained SDS–PAGE gel (9 %) of microtubule preparations at 24 °C and 37 °C. Identical egg extracts were prepared and equal volumes of each respective sample from the 24 °C (A–E) or 37 °C (F–J) preparation are compared. A,F. First-cycle warm supernatant fluids; B,G, first-cycle warm (microtubule) pellet; C,H, first-cycle cold-insoluble protein; D,I, second-cycle warm supernatant fluids; E,J, second-cycle warm (microtubule) pellet. The microtubule-assembly buffer for these experiments contained 100 mM-Pipes, pH 6·90, 1 mM-Mg_2SO_4, 4 mM-EGTA, 1 mM-GTP.

to obtain microtubules that were both calcium- and cold-labile and contained microtubule-associated components presumed important for mitosis. In this report, we describe: (1) a new method for the self-assembly of large quantities of egg microtubules in a unique microtubule assembly buffer that contains dimethyl sulphoxide (Me_2SO); (2) the composition and morphology of these MAP-containing microtubules: and (3) the labile assembly and disassembly of the egg microtubules *in vitro*.

Materials and methods

Preparation of MAP-containing microtubules

Microtubules were prepared from unfertilized sea-urchin eggs by cycles of temperature-dependent assembly and disassembly (Shelanski *et al.* 1973). Unfertilized eggs were obtained from the Pacific coast sea-urchin *Strongylocentrotus purpuratus* (Marinus, Inc., Westchester, CA) by intracoelomic injection of 0·5 M-KCl and were washed twice with artificial sea water (MBL formulation; Cavanaugh, 1956). The egg jelly was removed by washing once in 'isotonic 19:1' (500 mM-NaCl, 27 mM-KCl, 2 mM-EDTA, pH 7·80). The eggs were resuspended in an equal volume of lysis buffer (100 mM-piperazine-*N*,*N*′-bis[2-ethane sulphonic acid] (Pipes), pH 7·30, 1 mM-$MgSO_4$, 4 mM-ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2 mM-dithiothreitol, 1 mM-GTP, 1 mM-phenylmethylsulphonyl fluoride (PMSF), homogenized in a Dounce tissue grinder (5–6 passes), and centrifuged at 39 000 ***g*** (max.) (18 000 revs min^{-1} in a Beckman JA-20 rotor) at 2 °C for 45 min. The supernatant fluids (cytosolic extract) were made 8 % (v/v) in Me_2SO and 2 mM in GTP. In some cases, a GTP-regenerating system consisting of 10 mM-acetyl phosphate and 1 unit ml^{-1} acetate kinase was added before the extract was brought to 24 °C in a water-bath

for 45 min. The polymerized microtubules (first cycle) were pelletted by centrifugation at 39000 ***g*** (max.) for 30 min at 24°C. The microtubule pellet was resuspended in a microtubule-reassembly buffer (100 mM-Pipes, pH 6·90, 1 mM-$MgSO_4$, 1 mM-EGTA, 1 mM-GTP, no regenerating system) to 0·25 of the volume of the original cytosolic extract, and chilled on ice for 30 min. The depolymerized protein was centrifuged at 39000 ***g*** (max.) for 30 min at 2°C. The resulting cold supernatant fluids contained the solubilized microtubule proteins, which were reassembled into microtubules (second cycle) by raising the temperature to 30°C for 30 min. The microtubules were sedimented at 39000 ***g*** (max.) for 30 min at 30°C, resuspended in ice-cold microtubule-reassembly buffer (0·4 of the volume added for the previous cycle of disassembly), and processed for a total of three or four cycles of temperature-dependent assembly and disassembly. For comparison, MAP-containing microtubules were prepared by the taxol method as described (Vallee & Bloom, 1983).

Gel electrophoresis and protein determination

Proteins were resolved by sodium dodecyl sulphate–polyacrylamide gels electrophoresis (SDS–PAGE) with the discontinuous buffer system (Laemmli, 1970). The gels were fixed and stained with Coomassie Blue R-250 (Fairbanks *et al.* 1971) or silver (Merril *et al.* 1981). Protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as a standard.

Electron microscopy

Microtubules samples were diluted into microtubule-reassembly buffer containing 1 % glutaraldehyde and negatively stained with 1 % aqueous uranyl acetate on glow-discharged, carbon-coated Formvar grids. Microtubule pellets were also fixed at room temperature in microtubule-reassembly buffer containing 1 % glutaraldehyde and 0·5 % tannic acid, post-fixed in 0·5 % osmium tetroxide in 20 mM-sodium phosphate, pH 6·0, for 30 min on ice, stained *en bloc* with uranyl acetate, dehydrated, embedded and sectioned

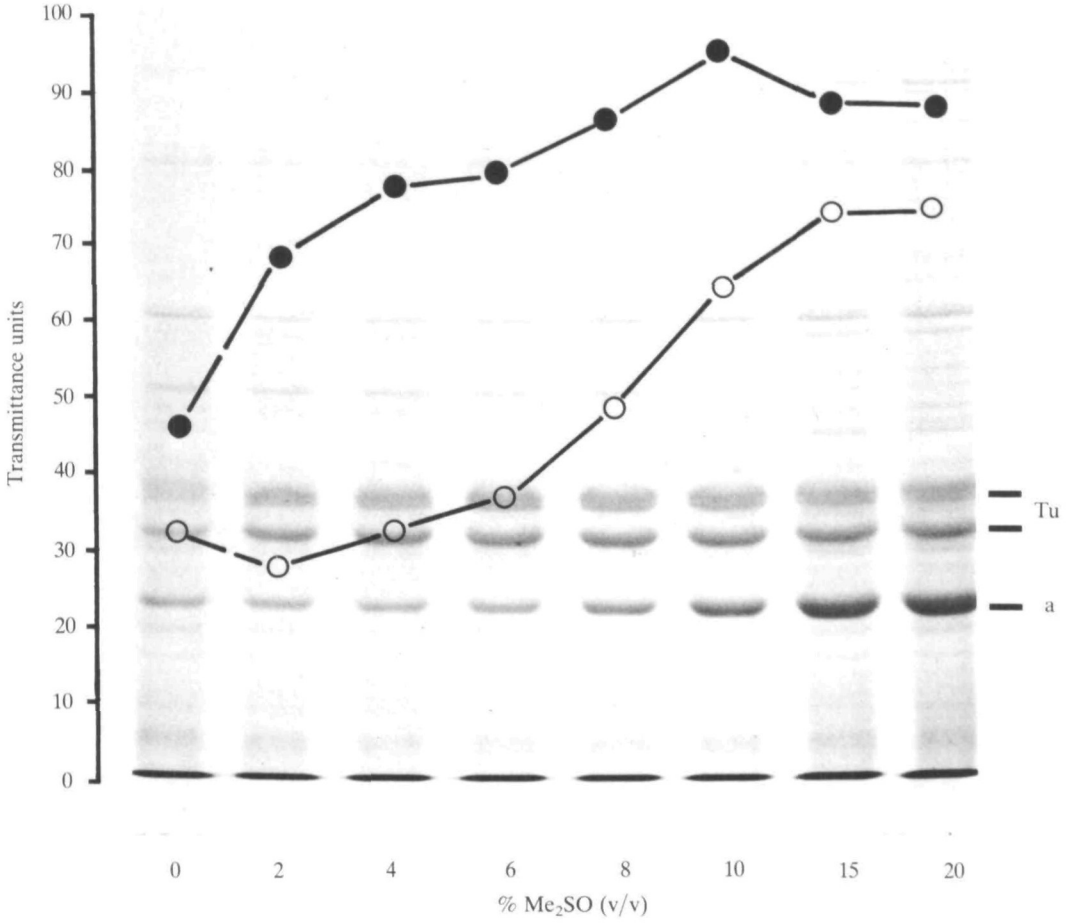

Fig. 2. Me_2SO promotes egg microtubule assembly at 24°C. Unfertilized sea-urchin eggs were homogenized in an equal volume of microtubule-assembly buffer that contains 100 mM-Pipes, pH 6·90, 1 mM-Mg_2SO_4, 4 mM-EGTA, 1 mM-GTP, and were centrifuged at 39000 ***g*** for 45 min at 2°C. Increasing concentrations of Me_2SO (0–20 %, v/v) were added to samples of the 39000 ***g*** supernatant fluids (cytosolic extract) that were incubated at 24°C for 45 min to assemble microtubules, and then centrifuged at 39000 ***g*** for 30 min to sediment the microtubules. The relative amount of tubulin (Tu) (●——●) and actin (a) (○——○) in each microtubule pellet was estimated by densitometry of SDS–polyacrylamide gels. The data are expressed as arbitrary transmittance units *versus* % Me_2SO.

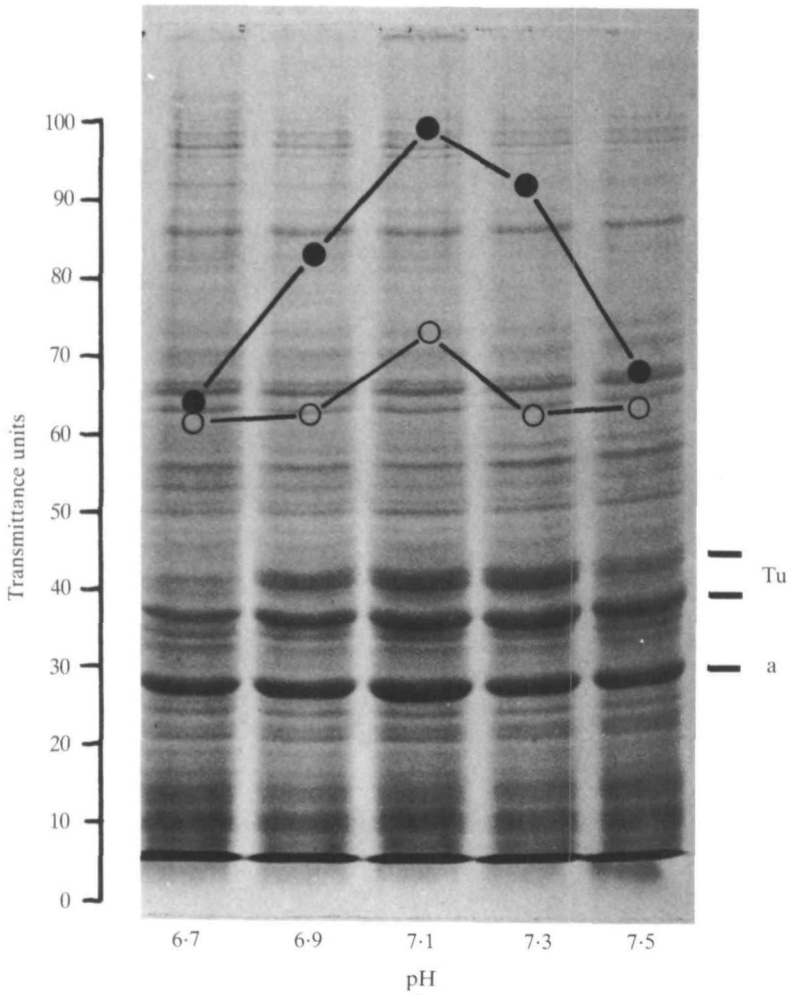

Fig. 3. pH optimum for microtubule assembly in unfertilized egg extracts. Unfertilized sea-urchin eggs were homogenized in an equal volume of microtubule-assembly buffer at either pH 6·7, 6·9, 7·1, 7·3 or 7·5, and centrifuged at 39000 ***g***. Me_2SO was added at a final concentration of 8% (v/v) and each preparation was warmed to 24°C for 45 min. The microtubule protein was sedimented by centrifugation and analysed by densitometry of SDS–polyacrylamide gels. The relative amount of tubulin (Tu) (●——●) and actin (a) (○——○) present in each microtubule pellet is expressed as arbitrary transmittance units *versus* the pH of the microtubule-assembly buffer.

for electron microscopy as described (Suprenant & Rebhun, 1984*a*). Samples were examined in a Phillips EM 300 at an accelerating voltage of 60 keV.

Results

Temperature restrictions for egg microtubule assembly

Numerous unsuccessful attempts to assemble microtubules from unfertilized egg extracts have prompted recent investigators to conclude that it is impossible to do so (Scholey *et al.* 1984). Others have suggested that there might be a specific inhibitor of microtubule assembly in unfertilized eggs or a storage form of tubulin that is destroyed, released or compartmentalized upon fertilization (Weisenberg, 1973; Bryan, 1975; Bryan *et al.* 1975). In addition, it appears equally plausible that the conditions necessary to promote egg tubulin assembly in a marine poikilotherm are not equivalent to those required for mammalian brain tubulin. With the latter possibility in mind, a new method for sea-urchin egg microtubule assembly was developed. Solution conditions were chosen to maximize the yield of microtubule protein and minimize the assembly of actin (Kane, 1975), and within feasible limits to remain close to the physiological temperature, pH and ionic composition of the egg.

Over 20 years ago, Farmanfarmaian & Giese (1963) reported that there is a rather sharp upper temperature tolerance for the purple sea-urchin *S. purpuratus*. Although the normal environmental temperature for growth of these organisms is 7–15°C, they reported that the sea-urchins remained normal; that is, they could climb, feed and right themselves, for up to 50 days at 23·5°C. In contrast, the urchins became limp

and unresponsive after 2 days at 24·5°C, and were dead after 24 h at 25°C.

To examine whether the higher temperatures used to assemble vertebrate brain tubulins were inhibitory to polymerization of sea-urchin egg tubulin, identical egg extracts were prepared and warmed to 24°C or 37°C. Microtubules were readily formed at 24°C but not at 37°C as assayed by negative staining for electron microscopy. Fig. 1 shows that tubulin, a major component of microtubules, was found in the 24°C preparations but not in the 37°C samples. The microtubules assembled at 24°C were cold-labile and could be depolymerized and reassembled during a second cycle of polymerization. In contrast, no microtubules and a cold-insensitive sedimentable aggregate of protein formed at 37°C. The upper temperature limit for obtaining a preparation of cold-labile microtubules was found to be 28–30°C; at temperatures higher than this cold-stable aggregates were formed.

Solvent conditions for microtubule assembly

To obtain quantities of microtubule protein suitable for biochemical analysis, the ionic strength, pH and solvent composition of the assembly buffer were varied to obtain optimum polymerization. Estimates of the amount of sedimentable actin and tubulin formed during the first cycle of polymerization were obtained by densitometry of polyacrylamide gels. Fig. 2 shows how microtubule assembly was promoted by the addition of dimethyl sulphoxide (Me_2SO), a solvent known to induce the assembly of 6 S brain tubulin by lowering the free energy of polymerization (Himes *et al.* 1977). Although microtubule assembly occurred in the absence of Me_2SO, polymerization was dramatically increased by the addition of Me_2SO at 24°C. In addition to increased quantities of microtubule protein, the amount of sedimentable actin was greatly increased by the presence of Me_2SO in the assembly mixture.

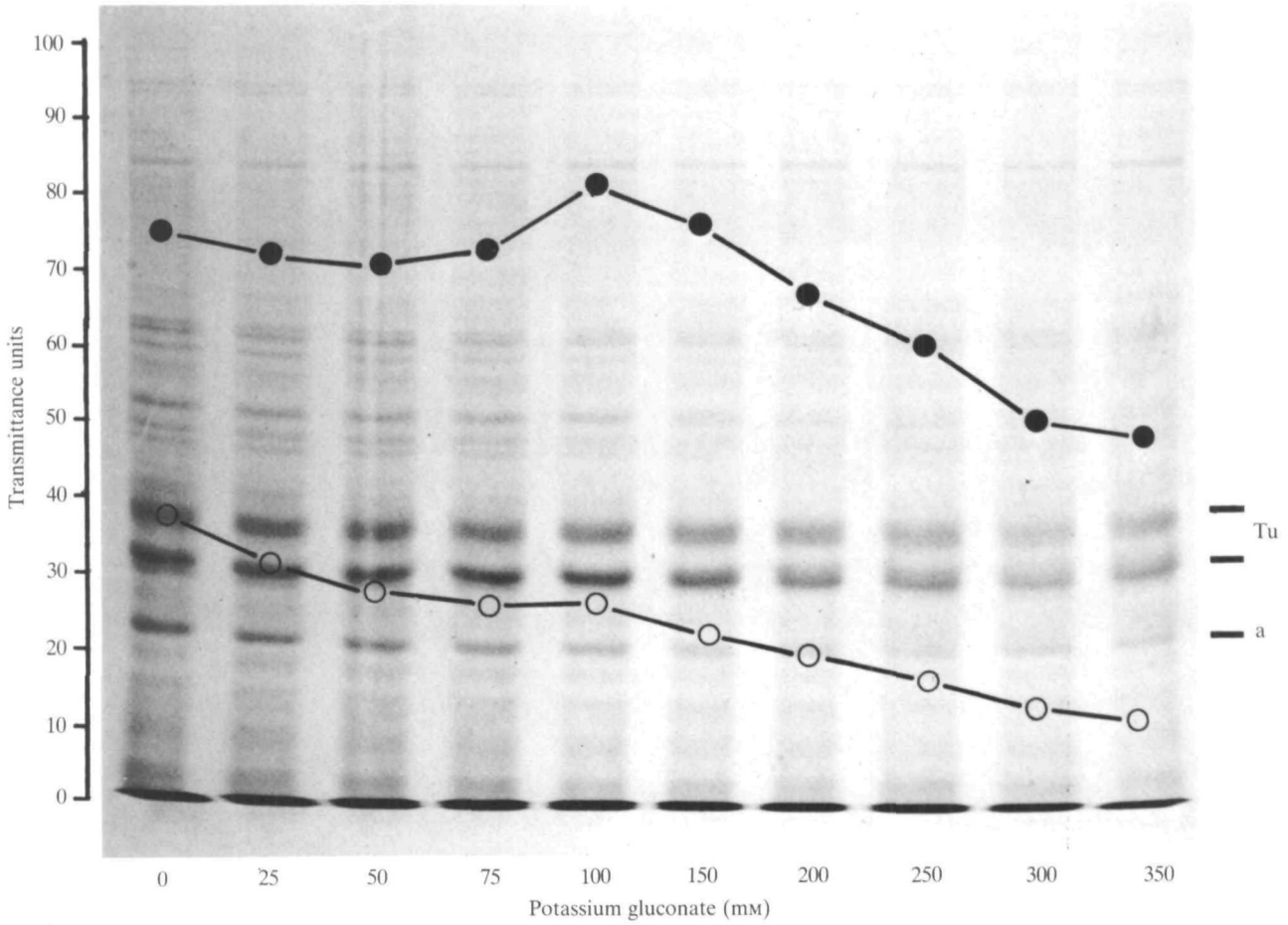

Fig. 4. Ionic strength optimum for microtubule assembly in unfertilized egg extracts. Unfertilized eggs were homogenized in an equal volume of microtubule-assembly buffer at pH 7·1 and centrifuged at 39 000 ***g*** for 45 min at 2°C. The cytosolic extract was made 8% (v/v) in Me_2SO, and increasing concentrations of potassium gluconate (0–350 mM final). The relative amounts of tubulin (Tu) (●——●) and actin (a) (○——○) in the microtubule pellets were estimated by densitometry of SDS–polyacrylamide gels and are expressed as arbitrary transmittance units *versus* mM-potassium gluconate.

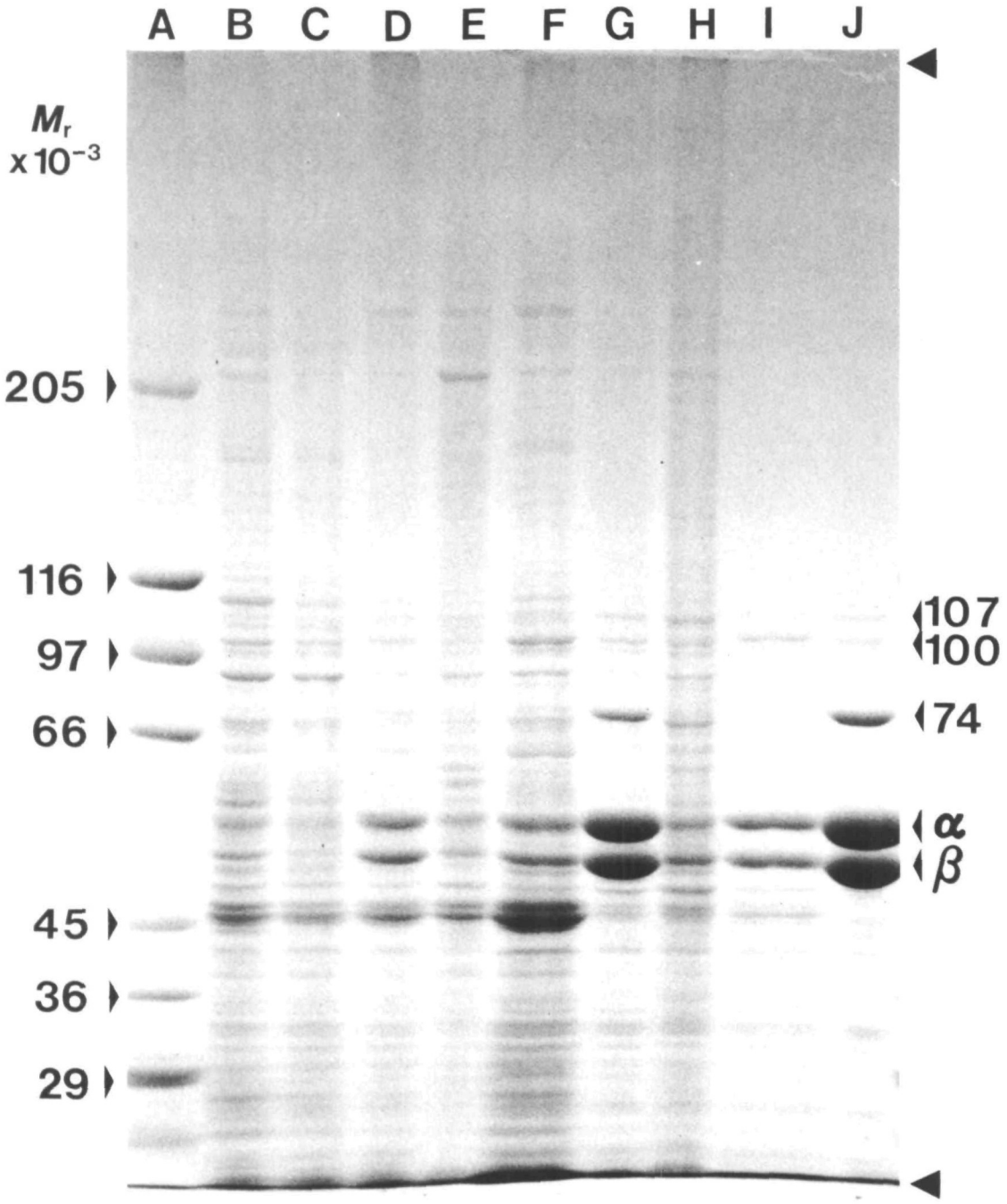

Fig. 5. Stages in the purification of sea-urchin egg microtubules directly from cytosolic extracts. Coomassie Blue-stained SDS–polyacrylamide gel containing 8 M-urea and a 4% to 16% gradient in acrylamide. Microtubule protein was isolated by three cycles of temperature-dependent assembly and disassembly as described in Materials and Methods. A. Molecular weight standards ($\times 10^{-3}$); B, cytosolic extract; C, first-cycle warm supernatant fluids; D, first-cycle microtubule pellet; E, first-cycle cold-insoluble pellet; F, second-cycle warm supernatant fluids; G, second-cycle microtubule pellet; H, second-cycle cold-insoluble pellet; I, third-cycle warm supernatant fluids; J, third-cycle microtubule protein. Large arrowheads mark the top of the gel and the dye front.

Fertilization of sea-urchin eggs leads to a transient rise in intracellular free Ca^{2+} and a permanent rise in intracellular pH from 6·8–7·2 for *Lytechinus pictus* and 7·0–7·4 for *S. purpuratus* (Johnson *et al.* 1976; Shen & Steinhardt, 1978; Dube *et al.* 1985). The increase in intracellular pH is necessary for the activation of protein synthesis (Grainger *et al.* 1979; Dube & Epel, 1986) and DNA synthesis (Whitaker & Steinhardt, 1981), as well as the polymerization of microtubules in these eggs post-fertilization (Schatten *et al.* 1985). Egg extracts were prepared in assembly buffer ranging in pH from 6·7 to 7·5 in order to see if

changes in pH would alter the amount of microtubule protein obtained *in vitro*. The results from these experiments are illustrated in Fig. 3. The largest amount of tubulin was sedimented from extracts prepared at pH 7·1–7·3 in the presence of 8 % Me_2SO at 24°C. Relatively little tubulin sedimented at the extreme pH values of 6·7 and 7·5.

Finally, the extent of microtubule assembly was determined as a function of ionic strength at pH 7·10, in the presence of 8 % Me_2SO at 24°C. The amount of tubulin pelleted was determined in the presence of increasing concentrations of potassium gluconate (Suprynowicz & Mazia, 1985). The amount of tubulin obtained (Fig. 4) was relatively constant over the range of 0–150 mM-potassium gluconate; only higher concentrations 200–350 mM brought about a significant reduction in the amount of tubulin pelletted. Since potassium gluconate did not significantly increase the amount of polymer isolated, it was not included in further purification schemes. Higher ionic strengths also brought about a decline in the amount of sedimentable actin and other unidentified proteins found in these first-cycle microtubule preparations.

Purification of microtubules and MAPs

Methods were developed for the temperature-dependent assembly and disassembly of egg tubulin in order to isolate microtubule-associated components that may be important for the structural, functional or metabolic functions of microtubules during mitosis. Egg microtubules with associated proteins were purified by cycles of temperature-dependent assembly and disassembly as described in Materials and Methods and the polypeptide composition of the assembled microtubules is shown in Fig. 5. The major proteins in the first-cycle microtubule pellet are alpha and beta-tubulin as well as actin. The microtubules formed are cold-labile and are readily depolymerized after resuspension in ice-cold microtubule-reassembly buffer. In fact, greater than 80 % of the polymer for each cycle of polymerization is cold-labile. By the third cycle of assembly, the microtubules are composed of tubulin and several presumptive MAPs. The major components of these third-cycle microtubules are alpha and beta-tubulin, and a major polypeptide of M_r 74 000 (74K). Densitometry of the third-cycle microtubule preparation (Fig. 6) indicates that the 74K polypeptide comprises 11 % of the microtubule

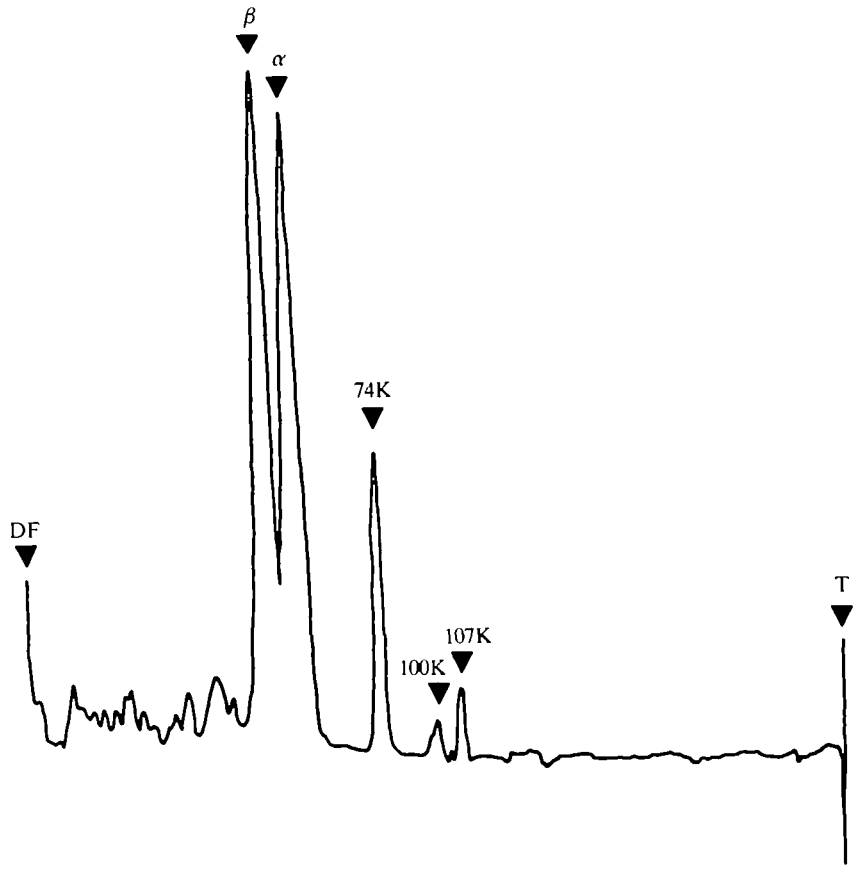

Fig. 6. Densitometry of third-cycle microtubule protein prepared as for Fig. 5, lane J. The top (T) of the gel and dye front (DF) are marked with arrows, as are the major polypeptides of third-cycle microtubules.

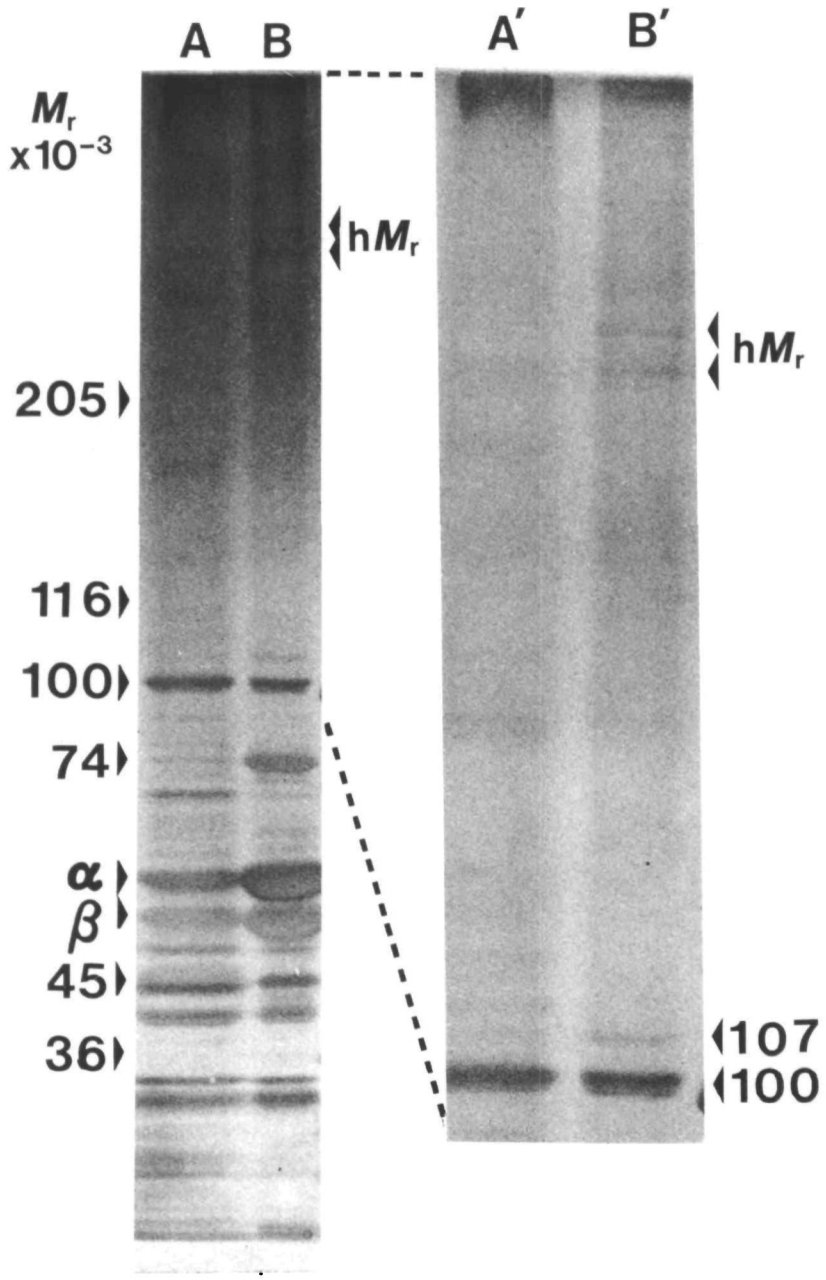

Fig. 7. Minor polypeptide components of third-cycle microtubules illustrated by silver-staining an SDS–polyacrylamide gel prepared identically as described for Fig. 5. A,A′. Third-cycle warm supernatant fluids; B,B′, third-cycle microtubule protein. hM_r, high molecular weight.

protein by mass and is present in a molar ratio of 0·18 (3 mol of 74K MAP/17 mol tubulin dimer). In addition to the 74K polypeptide, there are smaller amounts of a 100K doublet (<2% by mass) and a 107K polypeptide (<1% by mass) and trace quantities of high molecular weight proteins visible only by silver staining (Fig. 7).

Fig. 8 shows that the 74K polypeptide in the cycle-purified preparations migrates with an apparent molecular weight of 77 000 on a straight 9% acrylamide gel and with an apparent M_r of 80 000 on a 7·5% acrylamide gel (data not shown). The reason for this anomalous migration is not known. Nevertheless the 74K (alias 77K or 80K) polypeptide comigrates with the 77K taxol–MAP previously shown to be a component of sea-urchin microtubules *in vivo* (Vallee & Bloom, 1983; Bloom *et al.* 1985). The yield of microtubule protein in the two-cycle preparation is equivalent to that obtained with taxol and was found to be 6–8 mg/20 ml of packed eggs.

Both taxol- and cycle-purified microtubules illustrated in Fig. 8 were prepared in the absence of the proteolytic inhibitor PMSF. While the tubulin yields were the same in the presence and absence of PMSF,

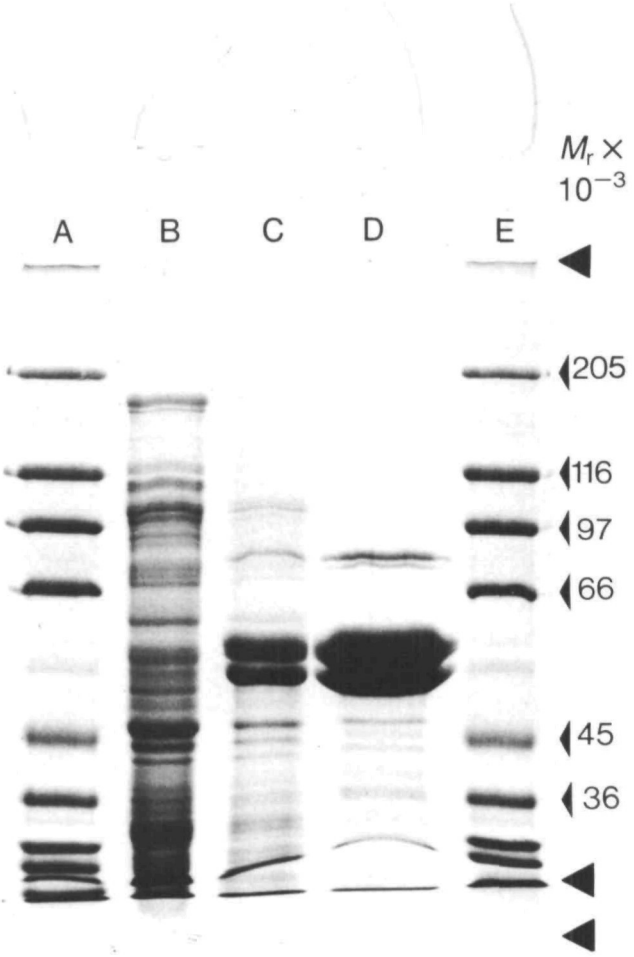

Fig. 8. Comparison of taxol–MAPs and cycle-purified MAPs prepared in the absence of the proteolytic inhibitor PMSF. A,E. Molecular weight standards ($\times 10^{-3}$); B, cytosolic extract depleted of microtubules during taxol-induced assembly; C, microtubule pellet obtained by taxol-induced assembly; D, second-cycle microtubule pellet. Larger arrowheads point to the top of the gel, the dye front and the bottom of the gel.

the amounts of presumptive MAPs were not, Compare the amount of 74K polypeptide (11 %) in Fig. 5 to the amount in Fig. 8 (3–4 %). In addition, the high molecular weight polypeptides usually found in the taxol–microtubule preparations in the presence of proteolytic inhibitors are not found associated with microtubules prepared in the absence of PMSF. This indicates that the sea-urchin MAPs are very sensitive to endogenous proteolytic activity in the egg extracts.

When microtubules isolated by three cycles of assembly were fixed and examined by electron microscopy, it was found that the microtubules were coated with globular projections rather than the filamentous projections frequently observed on MAP-containing microtubules (Fig. 9). The globular projections are found in microtubule preparations that have been fixed and negatively stained with uranyl acetate as well as in preparations that have been embedded and thin-sectioned. In thin sections, the globular projections (average diameter 24 nm) are observed to be attached to the wall of the microtubule *via* a thin stalk (average length 12 nm). The periodicity of the projections along the wall of the microtubule in negatively stained preparations is 37·5 ± 4 nm. On the basis of the electrophoretic composition of the microtubules, the globular projection is believed to be composed, at least in part, of the 74K polypeptide.

Polymerization of assembled microtubules

The assembly of egg microtubule protein at 24 °C was monitored turbidometrically (Fig. 10) and was found to proceed normally towards a steady-state plateau. The assembled microtubules, which are verified by negative staining for electron microscopy, are both calcium- and cold-labile. Following the addition of 20 μM-Ca^{2+}, approximately 32 % of the polymer depolymerized and re-established a new steady state. The remaining polymer was completely depolymerized by incubation on ice for 5 min.

The critical protein concentration (Ccr) for egg microtubule assembly was determined by a quantitative sedimentation assay (Johnson & Borisy, 1977). The steady-state distribution of polymer (filled circles) and monomer (open circles) is shown in Fig. 11. The critical protein concentration at 24 °C was found to be 0·11 mg ml^{-1}. Above this protein concentration, the amount of protein in the assembled polymer fraction is proportional to the total protein concentration, while the monomer concentration is nearly equivalent to the critical protein concentration. The slope of the monomer line is highly indicative of a large proportion of active microtubule protein (greater than 98 %).

Discussion

The spontaneous assembly of microtubules in cytosolic extracts prepared from unfertilized sea-urchin eggs was a success primarily because of the temperature and pH chosen for polymerization. The addition of Me_2SO to the assembly mixture is not required for egg microtubule assembly, but is included to increase the yield of microtubule protein obtained by approximately six- to tenfold. In addition, the proteolytic inhibitor PMSF is included to increase the amount of MAPs that coassembles with egg tubulin.

Sea-urchin egg microtubules are readily formed in extracts warmed to 24 °C, but not in extracts warmed to temperatures greater than 28–30 °C. This is not surprising since the organism usually lives in Pacific coast waters ranging in temperature from 7–15 °C, and cannot survive more than 24 h at 25 °C (Farman-

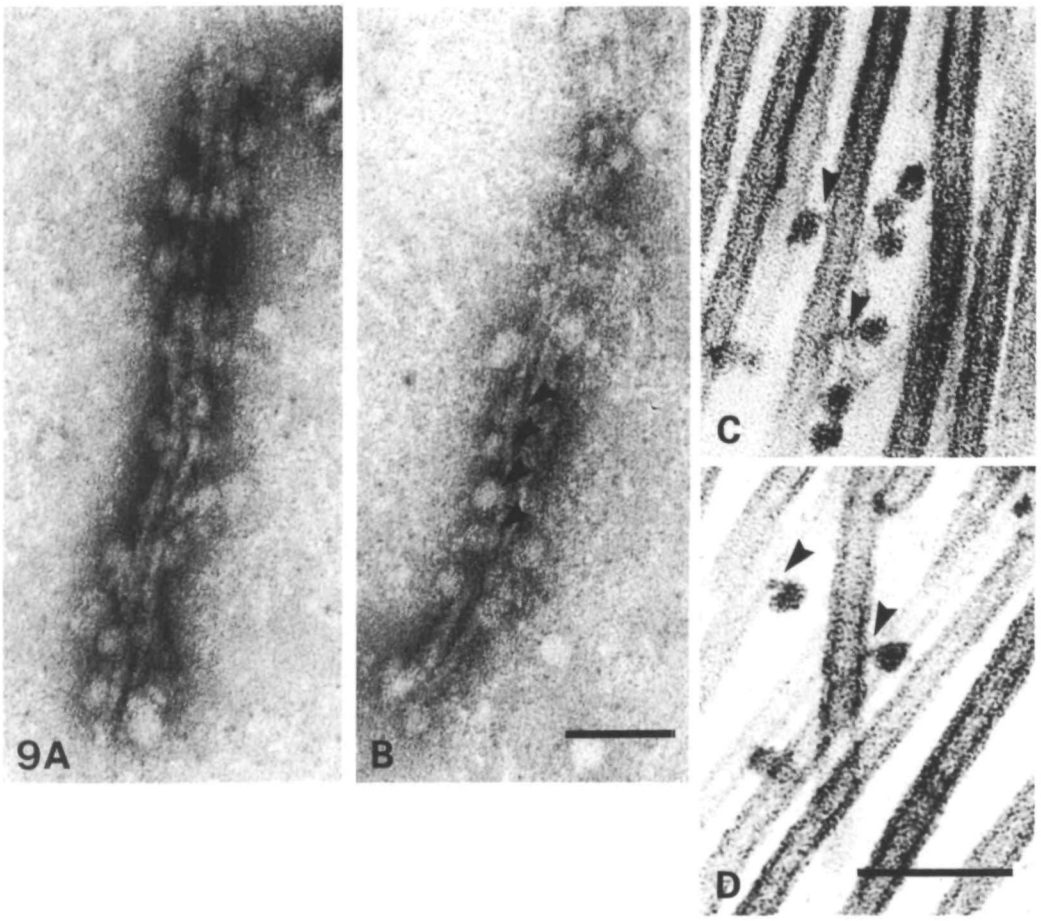

Fig. 9. Projections on sea-urchin egg microtubules. Microtubules were prepared (as for Fig. 5, lane J) from unfertilized sea-urchin eggs. The microtubules are decorated with globular projections when examined by negative staining (A,B) or by thin-sectioning (C,D) for electron microscopy. The periodic projections (arrowheads) appear to be composed of a globular head and a thin stalk that attaches to the microtubule. Bar, 100 nm.

farmaian & Giese, 1963). Once the microtubules have been assembled in the unfertilized egg extracts and sedimented out of the egg extracts they can be cold-depolymerized and reassembled at temperatures greater than 30°C. The requirement for a lower polymerization temperature is therefore only necessary during the initial cycle of assembly.

The pH necessary for optimum yields of microtubules in egg extracts was found to be 7·1–7·3. It is not known whether egg tubulin assembles to a greater extent or more efficiently at this pH, or whether the tubulin was released from an inhibitory complex by raising the pH of the extract above the pH of the unfertilized egg cytoplasm. Fertilization of sea-urchin eggs triggers the alkalinization of the cytoplasm by 0·4 pH unit (Johnson *et al.* 1976; Shen & Steinhardt, 1978). This increase in cytoplasmic pH has been shown to promote the formation of actin filaments in the sea-urchin egg cortex (Begg & Rebhun, 1979) and it would be significant if similar effects were observed on microtubule assembly. Schatten and coworkers (1985) recently showed that the pH change that occurs upon fertilization is necessary for microtubule polymerization *in vivo*. At the molecular level, the rise in internal cytoplasmic pH could release an inhibitor molecule from the tubulin dimer, enabling microtubule polymerization to occur in the presence of nucleation sites or assembly-promoting factors (Bryan, 1975). The tubulin would be stored or compartmentalized in the unfertilized egg by its association with the inhibitor at pre-fertilization pH values. Alternatively, an increase in cytoplasmic pH could increase the rate or extent of microtubule polymerization. Unfortunately, the pH optimum for microtubule assembly is reported to be around pH 6·4 (Gaskin *et al.* 1974; Regula *et al.* 1981); alkaline pH increases the Ccr and the rate of microtubule disassembly (Regula *et al.* 1981). Moreover, since purified egg tubulin assembles very well at the pre-fertilization pH of 6·80 (Suprenant & Rebhun, 1983; Detrich & Wilson, 1983), it is intriguing to speculate that the sea-urchin egg contains a factor, similar in principle to the actin-inhibitor protein profilin (Pollard & Cooper, 1986), that prevents the assembly of microtubules at

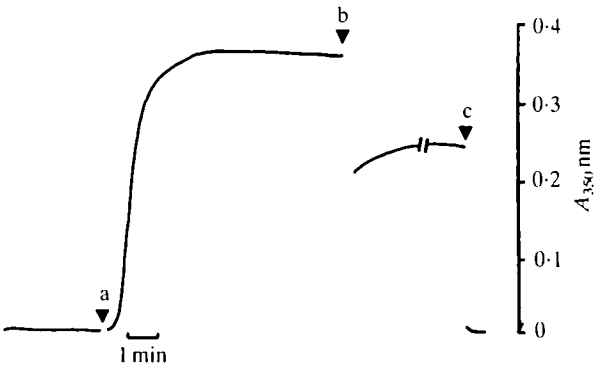

Fig. 10. Labile assembly and disassembly of sea-urchin egg microtubules. Microtubules were prepared (as for Fig. 5, lane 1) and diluted to 2·2 mg ml^{-1} in microtubule-assembly buffer containing 100 mM-Pipes, pH 6·90, 1 mM-Mg_2SO_4, 1 mM-EGTA at 24°C. The sample was made 1 mM in GTP (at a) and the assembly to steady state was monitored turbidometrically. A sample of $CaCl_2$ was added (at b) such that the calculated free calcium concentration was 20 μM. At this free calcium level approximately 32% of the microtubule preparation (by mass) was depolymerized and after 30 min the preparation established a new steady-state plateau (at c). The remaining polymer was rapidly disassembled by a 5-min incubation on ice, and the turbidity decreased to zero.

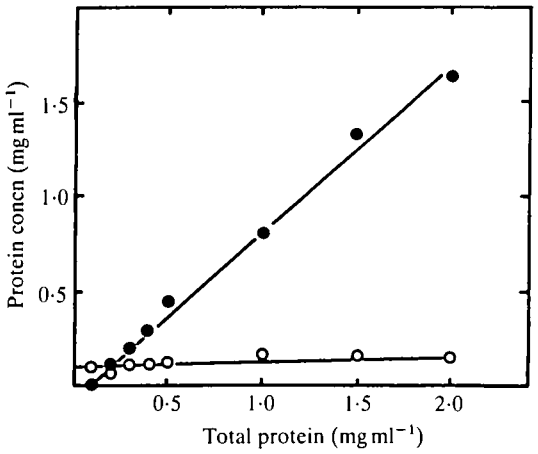

Fig. 11. Quantification of egg microtubule assembly at 24°C. The extent to which unfertilized egg tubulin assembles was determined by a quantitative sedimentation assay (Johnson & Borisy, 1977). Data were plotted by linear regression analysis in the form of protein concentration of subunit (○——○) or polymer (●——●) (ordinate) *versus* total protein concentration (abscissa). Extrapolation of the subunit concentration to infinite dilution indicates a critical protein concentration of 0·11 mg ml^{-1} at 24°C.

low pH by forming a stable complex with the tubulin dimer.

The preparation obtained by cycling is similar in many respects to the taxol–microtubule preparations (Vallee & Bloom, 1983; Scholey *et al.* 1984). Using Me_2SO to promote assembly, the yield of microtubule protein after two cycles of assembly was 6–8 mg per 20 ml eggs. Scholey and coworkers (1984) have reported nearly identical yields of microtubule protein using taxol (2–5 mg per 10 ml eggs). The microtubules obtained by both preparations are structurally intact and are composed of tubulin and MAPs. The major MAP in the cycled preparation is a 74K (77K or 80K, depending on % acrylamide in gel) polypeptide that comigrates with the 77K taxol MAP (Vallee & Bloom, 1983) or 80K taxol MAP (Scholey *et al.* 1984) and is probably identical to the 80K spindle MAP (Keller & Rebhun, 1980; Rebhun *et al.* 1982). Because of the anomolous migration of these MAPs on SDS–PAGE, it is suggested that investigators refer to the egg MAP as a 77K MAP after Vallee & Bloom (1983) and the spindle MAP as an 80K MAP after Keller & Rebhun (1980), until the 77K egg and 80K spindle MAP are shown to be one and the same.

The molar ratio of the 77K egg MAP to the 100K tubulin dimer in the three-cycle preparations is 0·18. This is greater than the molar ratio of 77K MAP to tubulin dimer in the taxol preparations (reported as 15 mol 80K per 130 mol tubulin dimer) (Scholey *et al.* 1984). Using monoclonal antibodies, Bloom and coworkers (1985) have shown that the 77K MAP is a component of mitotic microtubules, as well as cytoplasmic microtubules in differentiated sea-urchin coelomocytes. Our new preparation for obtaining MAP-containing microtubules from unfertilized sea-urchin eggs is therefore a valuable preparation for the isolation and study of sea-urchin MAPs.

Although it has not been directly shown in this preparation, it appears that the 77K MAP stimulates egg microtubule assembly at a lower Ccr value than egg tubulin alone. Egg tubulin purified by anion-exchange chromatography will assemble at 24°C at a Ccr of 0·3 mg ml^{-1} (Suprenant & Rebhun, 1983), while the MAP-containing microtubules (this report) assembles at a threefold lower protein concentration (Ccr of 0·1 mg ml^{-1}). Similarly, Scholey *et al.* (1983) reported that the 80K taxol–MAP stimulates the assembly of brain tubulin *in vitro*.

There are significant differences between the cycle-purified microtubules and the taxol–microtubules. The cycle-purified microtubules contain fewer high molecular weight MAPs shown to be present in the taxol–microtubule preparations, suggesting that these proteins have a much lower affinity for microtubules in the absence of taxol under our buffer conditions. Moreover, the morphology of the MAPs appears to be different in the cycle-purified and taxol preparations. The taxol-assembled microtubules are coated with fuzzy or filamentous projections (Vallee & Bloom, 1983; Scholey *et al.* 1984) that often display an axial

periodicity of 38·6 ± 1·2 nm (Scholey *et al.* 1984). On the other hand, the cycle-purified microtubules are coated with numerous globular projections that are attached to the microtubules by a thin stalk. In some negatively stained preparations the globular projections have an obvious axial periodicity of 37·5 ± 4 nm. The striking asymmetry of the globular projection suggests that there are two functionally different domains on the MAP projection. The composition of the projection and stalk are not known but are thought to be composed, at least in part, of the major non-tubulin protein in these preparations, the 77K MAP.

Another significant difference between the taxol preparation and the new cycling method is the lability of the assembled microtubules to cold and calcium. Cycle-purified egg microtubules were extremely cold-sensitive. Between 80 and 90% of each microtubule preparation (by mass) rapidly disassembled within 2–3 min at 0–4°C. Moreover, micromolar levels of free calcium depolymerized over 30% of a 2 mg ml^{-1} egg microtubule preparation at 24°C. These MAP-containing microtubules are not nearly as calcium-sensitive as microtubules assembled from egg tubulin alone (Suprenant & Rebhun, 1984*b*). This is not surprising since it has been shown that MAPs stabilize brain microtubules against calcium depolymerization (Rebhun *et al.* 1980; Berkowitz & Wolff, 1981; Schliwa *et al.* 1981). These MAP-containing egg microtubule preparations will, therefore, be very useful for studies of the calcium regulation of egg microtubules. Particularly, since it has been postulated that localized changes in free calcium in the mitotic apparatus regulate the *in vivo* polymerization of microtubules and the subsequent chromosomal movements to the pole (Harris, 1978; Hepler *et al.* 1981).

Finally, we suggest that Me_2SO may be useful for promoting microtubule assembly in other systems where microtubule assembly has proved inextractable. Me_2SO can be added to cytosolic extracts in order to stimulate the assembly of microtubules that are subsequently found to be composed of MAPs and tubulin. And unlike taxol–microtubules, these microtubules are calcium- and cold-labile and can be used for dynamic studies of microtubule-associated mechanochemical factors, such as cytoplasmic dynein (Weisenberg & Taylor, 1968; Pratt, 1980; Pratt *et al.* 1980; Hisanaga & Sakai, 1983; Hollenbeck *et al.* 1984; Scholey *et al.* 1984; Asai & Wilson, 1985) and kinesin (Vale *et al.* 1985; Scholey *et al.* 1985).

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