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The Reconstitution of Microtubules from Purified Calf Brain Tubulin†

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ABSTRACT: The in vitro reconstitution of calf brain tubulin, purified by the method of Weisenberg et al. [(1968), *Biochemistry 7,* 4466–4479; (1970), *Biochemistry 9,* 4110–4116] as modified by Lee et al. [(1973), *J. Biol. Chem. 248,* 7253–7262], was successful in a medium consisting of 10^{-2} *M* sodium phosphate, 10^{-4} *M* GTP, and concentrations of magnesium ions ranging from 0.5 to 16 × 10^{-3} *M* at 37°. Filaments resembling native microtubules were formed. The filaments are in equilibrium with the associating species of tubulin and the equilibrium can be shifted to depolymerization by lowering the temperature to 20°. Filament formation is inhibited by calcium ions which also cause disassembly of the formed filaments. The effects of calcium ion can be reversed by the addition of [ethylenebis(oxyethylenenitrilo)]tetraacetic acid. The formation of filaments is favored by the presence of 3.4 *M* glycerol; only twisted abnormal filaments are observed in the presence of 1 *M* sucrose. The high molecular weight components observed in the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of many tubulin preparations were shown not to be essential for the formation of the filaments.

The initial description by Weisenberg (1972) of the reconstitution of microtubules from partially purified brain tubulin has been followed by a number of reports on this system (Borisy and Olmsted, 1972; Shelanski et al., 1973; Kirschner et al., 1974; Erickson, 1974). In all of these studies, the starting material was either partially purified protein or crude homogenate of brain protein. No studies on the reconstitution of microtubules from tubulin purified by the Weisenberg (Weisenberg et al., 1968; Weisenberg and Timasheff, 1970) procedure have been described, although the statement has been made in the literature that "the colchicine binding protein isolated by this method does not polymerize into microtubules" (Weingarten et al., 1974). The demonstration by Frigon et al. (Frigon et al., 1974; Frigon and Timasheff, 1975a,b) that calf brain tubulin purified by the Weisenberg procedure could undergo a self-association reaction in the presence of magnesium ions with the formation of closed ring polymers with a most probable molecular weight of 2.86 × 10^6 prompted us to investigate whether even larger, possibly filamentous, aggregates could be induced to form from the pure fully dissociated protein. A preliminary electron microscopic study having indicated that microtubules can indeed be reconstituted from highly purified calf brain tubulin (Lee et al., 1975a,b), a detailed study of the reassembly of tubulin was initiated and the results are reported in this paper.

Materials and Methods

Sodium dodecyl sulfate, purchased from Fisher Scientific Co., was recrystallized before use. Acrylamide was obtained from Bio-Rad Laboratories. Spectroquality glycerol was purchased from Matheson Coleman and Bell, and ultra pure sucrose (lot ZZ 1604) was from Schwarz/Mann. Uranyl acetate and [ethylenebis(oxyethylenenitrilo)]tetraacetic

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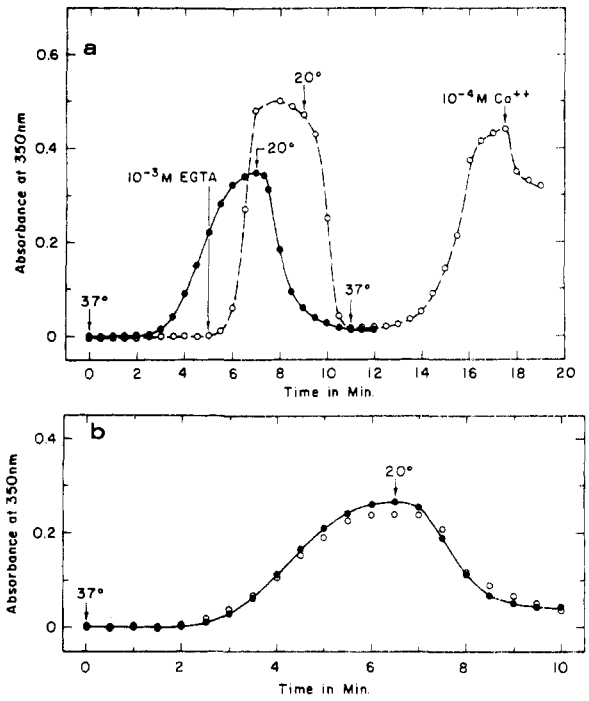

FIGURE 1: (a) Effect of temperature and calcium ions on the turbidity of calf brain tubulin in 3.4 *M* glycerol–PG–10^{-3} *M* EGTA–1.6×10^{-2} *M* $MgCl_2$. The protein and calcium ion concentrations were: (●) 2.5 mg/ml, none; and (○) 3.0 mg/ml, 2×10^{-5} *M*. (b) Effect of EGTA on the turbidity of tubulin in 3.4 *M* glycerol–PG–1.6×10^{-2} *M* $MgCl_2$ at 37°. The EGTA and protein concentrations were 10^{-3} *M* and 2.5 mg/ml, respectively. The symbols are (●) no EGTA and (○) with 10^{-3} *M* EGTA.

acid were purchased from Mallinckrodt Chemical Works and J. T. Baker Chemical Co., respectively.

Calf brain tubulin was prepared by a modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg and Timasheff, 1970; Lee et al., 1973; Frigon and Timasheff, 1975a). The purity of the protein was monitored by sodium dodecyl sulfate gel electrophoresis employing the procedure of Weber et al. (1972). Protein samples were denatured and reduced with 2-mercaptoethanol in a solution of 1% sodium dodecyl sulfate, and alkylated with iodoacetic acid. Reaction by-products were removed by dialysis against sodium dodecyl sulfate solution. The gels were stained with Coomassie Brilliant Blue G-250 and destained by diffusion.

Aggregation of tubulin was monitored by turbidity measurements (Gaskin et al., 1974a) and by electron microscopy. The assembly buffer, unless specified otherwise, consisted of 10^{-2} *M* sodium phosphate, 10^{-4} *M* GTP, 10^{-3} *M* EGTA,[1] 1.6×10^{-2} *M* $MgCl_2$, and 3.4 *M* glycerol at pH 7.0.

Turbidity measurements were performed at 350 nm on a Cary 14 recording spectrophotometer. The protein solutions were incubated at 37° in a water-jacketed cuvet which was thermostatically regulated by a Haake circulator.

The structural identity of the formed tubulin aggregates was monitored by electron microscopy. 10 μl of each protein sample was placed on a carbon-coated Formvar film cast on a copper grid of 400 mesh, which had been activated by a high voltage discharge in an atmosphere of isoamylamine (Dubochet et al., 1971). Adsorption was allowed to proceed for 1–3 min and the sample was then stained with 10 drops of 1% aqueous uranyl acetate for 15–30 sec. Excess stain was withdrawn from the grid with filter paper, and the sample was air-dried. Electron micrographs were taken with a Phillips Model 300 electron microscope and recorded on 35-mm film.

Fractionation sedimentation experiments were performed in a Beckman Model E analytical ultracentrifuge at 60000 rpm and 20°, using a single sector aluminum partition cell.

Results and Discussion

Since filament formation from brain extracts has been shown to be favored by the addition of glycerol, the sequestration of calcium ions by EGTA and by an increase in temperature (Weisenberg, 1972; Borisy and Olmsted, 1972; Shelanski et al., 1973), the reconstitution experiments were carried out at 37° in a medium consisting of PG buffer, 10^{-3} *M* EGTA, 1.6×10^{-2} *M* $MgCl_2$, and 3.4 *M* glycerol. Typical results are shown in Figure 1a. The turbidity, monitored at 350 nm, increased after an initial lag time, indicating the formation of large aggregates. Cooling to 20° led to a rapid decrease in turbidity.

Although Weisenberg (1972) reported that microtubule formation requires the addition of EGTA to sequester calcium ions, in our system, the addition of EGTA did not enhance the formation of large aggregates as shown in Figure 1b, essentially identical turbidities being reached at equilibrium for tubulin solutions of identical concentrations, whether EGTA was present or not. Addition of 2×10^{-5} *M* $CaCl_2$ to the protein solution, however, did inhibit the formation of aggregates since, in this case, heating to 37° did not lead to any increase in turbidity as shown by the open circles of Figure 1a. Subsequent addition of 10^{-3} *M* EGTA to the calcium ion containing solution, however, resulted in an increase in turbidity after heating to 37°. This indicates that the lack of the EGTA addition requirement in our system is due to a low level of endogenous calcium ions in our tubulin preparations. The formation of aggregates is a reversible reaction; the turbidity decreased either when the solution was made up to 10^{-4} *M* $CaCl_2$ or when it was cooled to 20°, as shown in Figure 1a. The turbidity could be restored again by heating to 37°.

The reversible aggregation of tubulin was inhibited by colchicine, since heating to 37° in the presence of 10^{-4} *M* colchicine did not lead to any increase in turbidity, monitored at 500 nm. Addition of 10^{-4} *M* colchicine to an aggregated protein solution at 37° was followed by a decrease in turbidity. This disruptive effect of colchicine could, in turn, be reversed by exposure to 350 nm.

Furthermore, the formation of reversible aggregates appears to require the presence of GTP, as indicated by the reversible increase in turbidity on heating to 37°, following the addition of 10^{-4} *M* GTP to a solution which had shown only irregular and irreversible increase in turbidity when it was heated in the absence of GTP.

The effect of tubulin concentration on the formation of aggregates is shown in Figure 2. There was little or no observable increase in turbidity with tubulin solutions at concentrations at or below 1.0 mg/ml. At higher protein concentrations, however, heating to 37° resulted in an increase in turbidity which reached a plateau after 6 to 7 min of heating, the plateau values of turbidity being proportional to the initial protein concentration, C_0, as shown in the inset of Figure 2. Such a concentration dependence of the aggre-

[1] Abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PG buffer, pH 7.0, 10^{-2} *M* sodium phosphate buffer containing 1×10^{-4} *M* GTP.

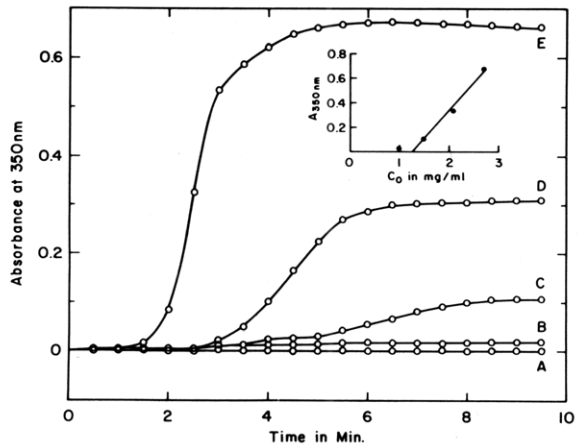

FIGURE 2: Effect of tubulin concentration on turbidity. The solvent was 3.4 *M* glycerol–PG–1.6×10^{-2} *M* $MgCl_2$–10^{-3} *M* EGTA. The protein concentrations were: (A) 0.3 mg/ml; (B) 1.0 mg/ml; (C) 1.5 mg/ml; (D) 2.1 mg/ml; and (E) 2.7 mg/ml. The inset is a plot of plateau turbidity values vs. initial protein concentration, C_0.

gation, with a "critical concentration", is characteristic of cooperative processes.

The structure of the aggregate formed was identified by electron microscopy. Typical results, shown in Figure 3A, indicate the presence of filaments. On higher magnification (Figure 3B) these are seen to have a regular structure. The diameter of the observed filaments was 29 ± 3 nm, similar to the dimensions of intact native microtubules (Olmsted and Borisy, 1973). The appearance of protofilaments, such as are seen in normal microtubules, is unmistakable. No filaments could be observed after cooling to 20° or below.

The results of these turbidity and electron microscopy experiments clearly show that microtubules can be reconstituted from tubulin purified by the Weisenberg method. The reconstituted structures are morphologically similar to intact native microtubules and they possess properties that are characteristic of microtubules, such as disruption of structure by low temperature, addition of calcium ions and of colchicine, as well as inhibition of filament formation by the presence of low concentrations of Ca^{2+} or of colchicine.

The identification of the tubulin aggregates as microtubules permits a more quantitative analysis of the results of Figure 2. The presence of a "critical concentration" suggests that the polymerization takes place as a helical condensation phenomenon, similar to that described by Oosawa and Higashi (1967) for the G-actin → F-actin reaction. In such a mechanism, the observed critical concentration, C_r, is equal within close approximation to the inverse of the association constant, K_p, for the addition of a monomeric unit to a helical, or tubular polymer. The value of C_r, found in the plot of Figure 2, is 1.2×10^{-5} *M* tubulin, giving $K_p = 8.3 \times 10^4$ l./mol, and an apparent free energy of polymer propagation, $\Delta G° = -7$ kcal/mol.

With the knowledge that microtubules can be reconstituted from highly purified tubulin, the solution requirements for this reaction were examined—namely, the effects of magnesium ions, glycerol, sucrose, and the need for preexisting nucleation centers. Glycerol and sucrose are known to stabilize microtubule protein (Frigon and Lee, 1972; Shelanski et al., 1973; Kane, 1962), and the formation of filaments has been reported in the presence of sucrose (Shelanski et al., 1973) and to be favored by glycerol (Rebhun et al., 1974). Furthermore, Weisenberg (1972) and Gaskin et al. (1974a) have shown that microtubules

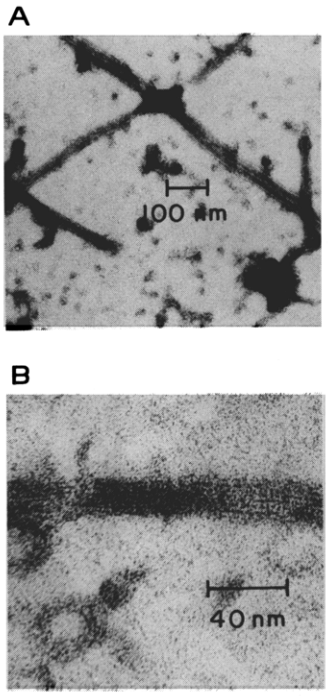

FIGURE 3: Electron micrographs of filaments formed by calf brain tubulin in 3.4 *M* glycerol–PG–1.6×10^{-2} *M* $MgCl_2$–10^{-3} *M* EGTA at 37°. The magnification factors are: (A) 50000×; (B) 122500×.

can be reconstituted from partially purified brain tubulin at magnesium levels as low as 5×10^{-4} *M*. The effects of varying these solvent components were investigated and the results are shown in Figure 4. Lowering the $MgCl_2$ concentration from 1.6×10^{-2} to 5×10^{-4} *M* or omitting glycerol from our reconstitution medium gave the results shown in Figure 4A and B. It is evident that tubulin purified by the Weisenberg procedure can be reconstituted to microtubules under these conditions, although some amorphous material is attached to them, and the frequency of observing filaments per unit grid area is lower than that in high magnesium concentration or in the presence of glycerol.

Figure 4C shows the results obtained when glycerol was replaced by 1 *M* sucrose in the reconstitution medium. It is evident that filaments are formed. However, these filaments are seen to be different from normal microtubules. They have a twisted structure and contain no observable protofilaments. Thus, although sucrose, just as glycerol, protects purified tubulin from denaturation (Frigon and Lee, 1972), it affects reassembly to microtubules in a different manner. The exact mechanisms of protein stabilization and enhancement of filament formation by glycerol and sucrose are not known. They seem to act through nonidentical nonspecific solvent interactions (Timasheff et al., 1975), the exact nature of which is currently under investigation in our laboratory.

It has been stated that microtubules cannot be reassembled from totally dissociated tubulin (5.8 S, 110000 mol wt) (Weingarten et al., 1974; Erickson, 1974), and that preexisting nucleation centers or other protein factors are required for this polymerization to take place. At least one of these protein factors has been identified with a fraction of tubulin preparations that can be seen in dodecyl sulfate polyacrylamide gel electrophoresis as high molecular weight components (Erickson, 1974; Gaskin et al., 1974b). Borisy and Olmsted (1972) have asserted that the essential nucleation centers present in the particulate fraction are

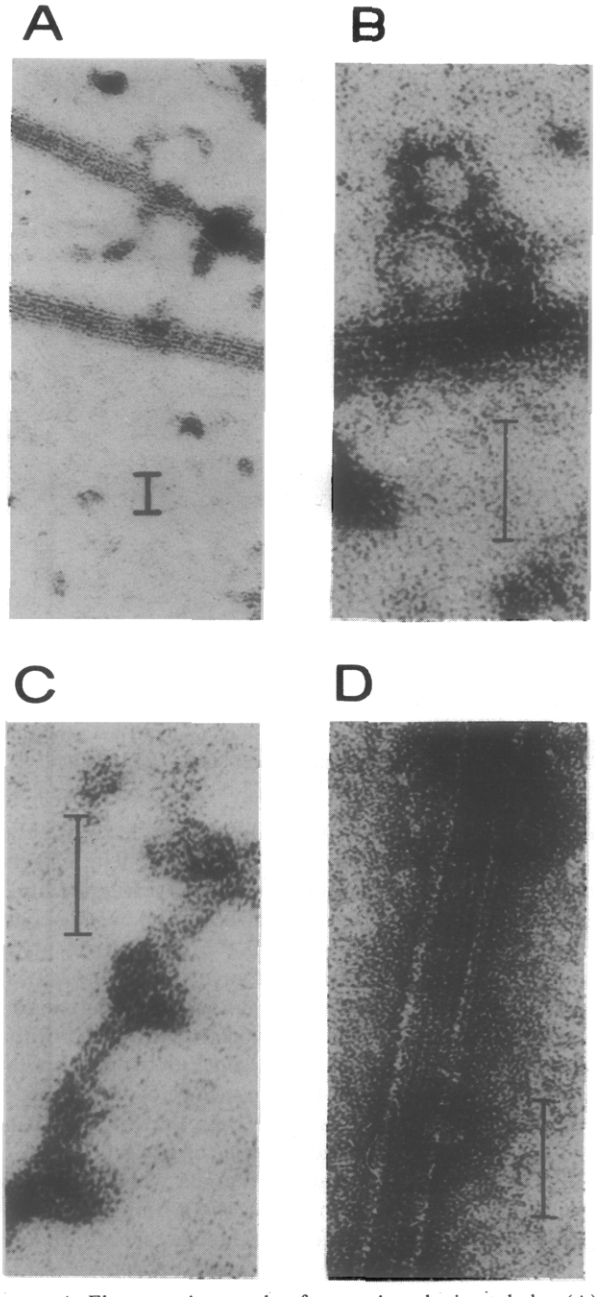

FIGURE 4: Electron micrographs of reconstituted microtubules. (A) In 3.4 *M* glycerol–PG–5 × 10^{-4} *M* $MgCl_2$–10^{-3} *M* EGTA at 37°. The magnification factor is 105000×. (B) In PG–1.6 × 10^{-2} *M* $MgCl_2$–10^{-3} *M* EGTA at 37°. (C) In 1 *M* sucrose–PG–1.6 × 10^{-2} *M* $MgCl_2$–10^{-3} *M* EGTA at 37°. (D) Tubulin solution after high-speed sedimentation. The solvent system is the same as in Figure 1a. The magnification factor for B–D is 325000×. The bars represent 50 nm.

discs with diameters similar to the cross-section diameter of intact microtubules. Kirschner et al. (1974), on the other hand, have stated that depolymerized microtubules can be repolymerized only from double rings, or spirals, with a molecular weight of 2.3 × 10^6 and a sedimentation coefficient of 36 S (Kirschner and Williams, 1974), the 5.8 S, 110000 mol wt tubulin dimer being incapable of repolymerization into microtubules and actually being different from subunits which can form 36S rings.

In order to check whether preexisting nucleation centers were present in our system, calf brain tubulin solutions, prepared by the Weisenberg procedure, were subjected to stringent tests of purity, even though it had been shown that

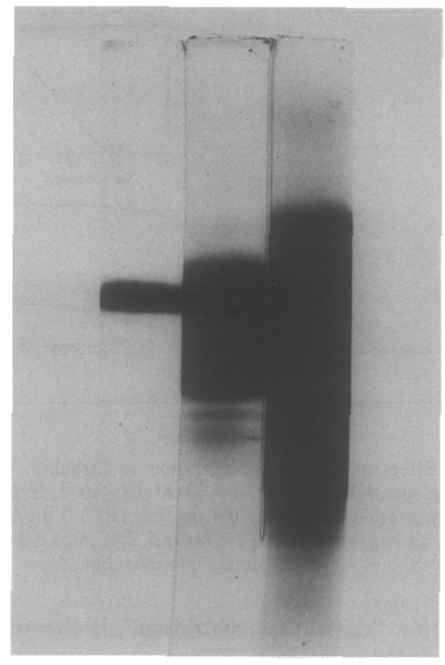

FIGURE 5: Sodium dodecyl sulfate polyacrylamide gels of tubulin. The loading protein concentration and identity of samples are: (A) 30 μg of tubulin purified by the Weisenberg procedure; (B) 160 μg of the same sample as (A); (C) 350 μg of tubulin after high-speed sedimentation.

the dodecyl sulfate polyacrylamide gels of such tubulin preparations have only a single band (Lee et al., 1973). Dodecyl sulfate gel electrophoresis experiments were, therefore, carried out at various loadings, with the results shown in Figure 5. Under normal protein loading concentrations, ca. 20–30 μg, there is only one band observable for the tubulin solution prepared by the present procedure. Increasing the loading concentration to ca. 160 μg revealed the presence of a very small amount of slowly moving components, considerably smaller than the contents reported for the preparations normally used in reconstitution experiments (Erickson, 1974).

In order to check whether preexisting nucleation centers were present in our system, calf brain tubulin solutions, prepared by the Weisenberg procedure, were subjected to stringent tests of purity, even though it had been shown that the dodecyl sulfate polyacrylamide gels of such tubulin mained in the compartment centripetal to the partition. The purity of the tubulin solution in this compartment of the cell was then checked by dodecyl sulfate gel electrophoresis. The results for such a sample, shown in Figure 5C, demonstrate that, even at a loading concentration of 350 μg, no high molecular weight components are observed. The ability of these tubulin solutions, highly purified by the sedimentation procedure, to reassemble into microtubules was tested by turbidity and electron microscopy, with the results shown in Figures 4D and 6. It is evident that heating of these tubulin solutions to 37° results in the formation of aggregates as shown in Figure 6. The aggregation reaction is reversible, as shown by the response of turbidity to temperature changes. Figure 4D shows that the aggregates formed are indeed microtubules. Thus, fully dissociated tubulin can be reassembled into microtubules in the absence of high molecular weight components detectable by dodecyl sulfate gel electrophoresis.

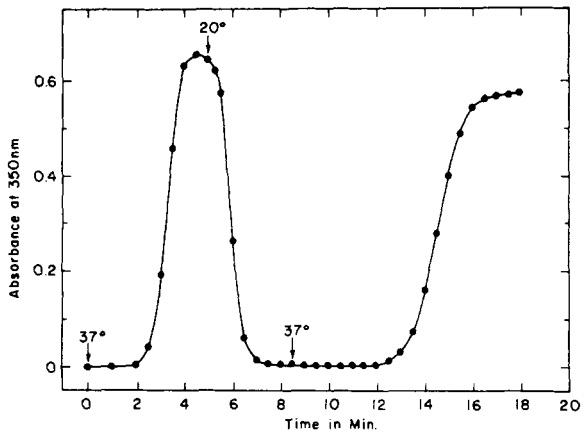

FIGURE 6: Effect of heating to 37° on the turbidity of a tubulin solution which had been sedimented in a partition cell. The solvent composition is the same as in Figure 1a.

In conclusion, the results of this study have shown that tubulin purified by the Weisenberg procedure can be reconstituted into microtubules. The formation of microtubules is favored by higher concentrations of magnesium ions and the presence of glycerol. Furthermore, in vitro microtubule assembly does not appear to require the presence of preexisting nucleation centers in the form of high molecular weight components.

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