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Reversible Dissociation of the $\alpha\beta$ Dimer of Tubulin from Bovine Brain†

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ABSTRACT: The reversible, concentration-dependent dissociation of the $\alpha\beta$ dimer of bovine brain tubulin (purified by phosphocellulose chromatography) has been demonstrated by equilibrium ultracentrifugation. The dissociation constant is approximately 8×10^{-7} M at 4.6 °C in PM buffer (0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid), 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 1 mM $MgSO_4$, 0.1 mM guanosine triphosphate, 2 mM dithioerythritol, at pH 6.9). This result was confirmed by observation of an appropriate dependence of the sedimentation coefficient of very dilute (<0.5 mg/mL) tubulin on its concentration. Small zone gel filtration experiments on Bio-Gel P-150 also demonstrated an increase in peak elution volume with decreasing column load concentration. Reversibility of the dissociation was demonstrated directly by sedimentation velocity and gel filtration experiments on tubulin reconcentrated from dilute solution by pressure ultrafiltration. Control experiments accompanying the sedimentation equilibrium experiments showed that this tubulin retained, under the conditions of the experiments, both its ability to form microtubules and more than 70% of its initial colchicine-binding activity.

The ordered assembly, maintenance, and disassembly of cytoplasmic microtubules appear to play critical roles in the functioning of eukaryotic cells (Porter, 1966; Olmsted & Borisy, 1973; Roberts, 1974; Snyder & McIntosh, 1976; Pipeleers et al., 1976; Yahara & Edelman, 1975; Edelman, 1976). An understanding of these roles will depend in part upon detailed knowledge of the properties of the major protein constituent and functional subunit of the microtubule, the tubulin dimer. This structure is probably an $\alpha\beta$ heterodimer of two related polypeptide chains (Bryan & Wilson, 1971; Ludueña et al., 1977) held together by noncovalent forces (Lee et al., 1973).

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In principle there should exist an equilibrium of the type

$$\alpha\beta \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \alpha + \beta$$

with a dissociation constant, K_D, given by

$$K_D = \frac{k_1}{k_{-1}} = \frac{C_\alpha C_\beta}{C_{\alpha\beta}}$$

where k_1 and k_{-1} represent the rate constants for the forward and reverse processes, and C_α, C_β, and $C_{\alpha\beta}$ are the concentrations of α and β chains and of the $\alpha\beta$ dimer. Whether this equilibrium is observable depends upon the magnitudes, both relative and absolute, of k_1 and k_{-1}. Apparently, no previous report of an observation of such a dissociation is to be found in the literature. We report here evidence obtained by three different methods that indicates that the $\alpha\beta$ dimer of bovine brain tubulin undergoes an observable and rapidly reversible dissociation into its subunits, and we present an estimate of the dissociation constant under one set of near-physiological conditions of pH and ionic strength. A preliminary report of some of this work has appeared (Detrich & Williams, 1977).

Materials and Methods

Reagents. Colchicine, Pipes,[1] EGTA, DTE, and GTP (type II-S) were obtained from Sigma. Colchicine (ring C, *methoxy*-^{3}H), 18.45 mCi/mmol, was purchased from New England Nuclear, Inc.

Experimental Conditions. All experiments were performed in a buffer of 0.1 M Pipes–KOH (pH 6.9), 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM GTP, 2 mM DTE (designated PM buffer) at temperatures between 4 and 6 °C, except where noted. The ionic strength of this buffer was approximately 0.236, and its density, determined pycnometrically, was 1.017 g/mL at 4.95 °C.

Tubulin Purification. Beef brains were obtained from freshly slaughtered animals, stored on ice, and used within 1 h after slaughter. Microtubule protein[2] was purified through two cycles of polymerization/depolymerization by the method of Shelanski et al. (1973) as modified by Berkowitz et al. (1977). Tubulin was separated from the microtubule-associated proteins (MAPs) by chromatography on phosphocellulose (Whatman P11) equilibrated with PM buffer (Weingarten et al., 1975; Sloboda et al., 1976; Himes et al., 1976; Herzog & Weber, 1977; Himes et al., 1977; Wehland et al., 1977). This phosphocellulose-purified (PC−) tubulin was either used immediately or stored at −196 °C by dropwise freezing in liquid nitrogen. Prior to use, material stored in liquid nitrogen was thawed rapidly, centrifuged at 5000*g* for 10 min to remove small amounts of denatured protein, and then chromatographed on a 0.9 cm by 25 cm column of Sephadex G-25 (Pharmacia) equilibrated with PM buffer. (Chromatography on G-25 was omitted for the gel filtration experiments described below.) Finally, the protein concentration was adjusted to the value required for a given experiment by dilution with PM buffer. Identical results were obtained from frozen and never-frozen material, and from several different microtubule preparations.

Protein Determinations. Tubulin concentrations were measured spectrophotometrically (an extinction coefficient at 280 nm of 1.20 mL/(mg·cm) was employed), or by the method of Bradford (1976) (an extinction coefficient at 595 nm of 0.891 mL/(mg·cm) was employed for the dye-reacted protein). Extinction coefficients were determined by the synthetic boundary technique of Babul & Stellwagen (1969). A value for the refractive index increment was derived from the data of Perlman & Longsworth (1948).

Electrophoresis. $NaDodSO_4$–polyacrylamide disc gel electrophoresis was performed on 7.5% cylindrical or slab gels according to the method of Laemmli (1970). Cylindrical gels used for quantitative studies were fixed and stained with 1% (w/v) Fast Green (Gorovsky et al., 1970) in 50% methanol–7% acetic acid (v/v) for 24 h (Berkowitz et al., 1977) and destained with 5% methanol–7% acetic acid. Percent purity of tubulin preparations was estimated by scanning the gels at 640 nm with a GCA/McPherson Model EU 701 spectrophotometer equipped with a linear transport. The amount of protein in a given electrophoretic band was determined by integration. Slab gels were stained with Coomassie Brilliant Blue R-250 (Berkowitz et al., 1977).

Colchicine Binding Assays. The binding of colchicine to tubulin was assayed by gel filtration to separate free colchicine from the colchicine–tubulin complex (Weisenberg et al., 1968; Wilson et al., 1974). A 1-mL aliquot of protein (0.5–3.0 mg/mL) was incubated with 100 μL of ^{3}H-labeled 1 mM colchicine (10–25 μCi/mL) for 1.5 h at 37 °C, applied to a PM-equilibrated 0.9 × 25 cm column of Sephadex G-25, and eluted with PM buffer. Recovery of the complex could be obtained within 15 min. Fractions (25 drop, ≃1.3 mL) were collected and protein concentrations were determined as described previously. Values of A_{280} were corrected for the absorbance contributed by the bound colchicine (ϵ_{280} = 4660 M^{-1} cm^{-1}). Radioactivity was assayed by liquid scintillation counting in a Packard Model 3330 Tri-Carb counter. Aliquots (0.5 mL) of each fraction were counted in 10 mL of ACS (Amersham/Searle). Observed counts/min were reduced to disintegrations/min by means of sample channels ratio quench correction.

Ultrafiltration. Some experiments required the reconcentration of a previously diluted tubulin sample. This was accomplished by the use of an Amicon Model 52 Ultrafiltration Cell and Diaflo PM30 membranes. Concentrated samples were degassed for 2 min in a vacuum desiccator, followed by centrifugation and Sephadex G-25 chromatography as described for material frozen in liquid nitrogen.

Gel Filtration Chromatography. PC-tubulin was chromatographed on a 0.9 cm × 65 cm column of Bio-Gel P-150 (Bio-Rad Laboratories) overlayed with 18 cm of Sephadex G-25. Samples (2 mL) were applied to the PM-equilibrated column and eluted with PM buffer. Sample concentrations applied to the column ranged from 0.076 mg/mL to 4 mg/mL. In all cases the diluted tubulin was incubated on ice for 1 h prior to gel filtration. Blue Dextran 2000 (2.0 mg/mL), catalase (2.5 mg/mL), BSA (4.0 mg/mL and 0.4 mg/mL), ovalbumin (1.9 mg/mL), and sperm whale myoglobin (1.8 mg/mL) were used to calibrate the column. To obtain precision in the measurement of eluant volumes, fractions were collected in tared test tubes and were then weighed to ±0.01 g. Although quantitatively difficult to interpret, the results of small zone gel filtration chromatography are useful for qualitatively demonstrating protein dissociation (Ackers, 1970). As the concentration of the column load decreases, the peak elution

[1] Abbreviations used: CBA, colchicine-binding activity; DTE, dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; HMW, high molecular weight; LMW, low molecular weight; MAPs, microtubule-associated proteins; $M_w(r)$, radial weight-average molecular weight; PC, phosphocellulose; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); $NaDodSO_4$, sodium dodecyl sulfate.

[2] In this paper microtubule protein refers to tubulin plus all the associated proteins (MAPs) which copurify with tubulin through cycles of in vitro assembly and disassembly. Tubulin refers specifically to the $\alpha\beta$ dimer of the microtubule.

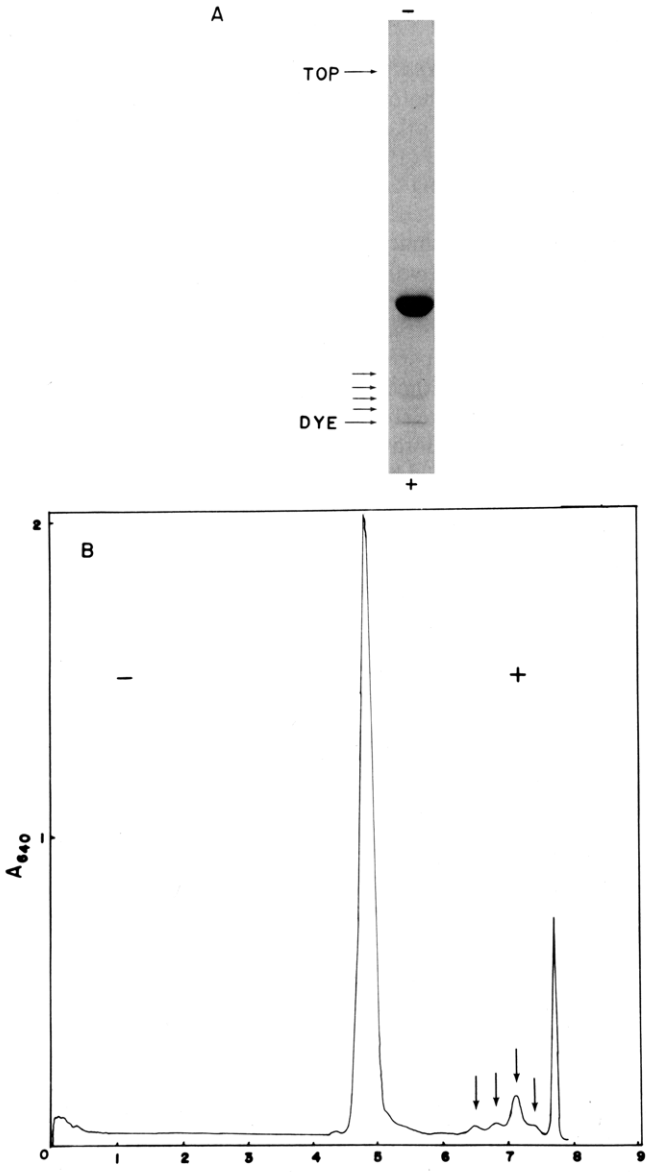

FIGURE 1: Electrophoretic analysis of PC-tubulin. (A) PC-tubulin (74 μg) was run on a 7.5% $NaDodSO_4$–polyacrylamide slab gel and stained with Coomassie Brilliant Blue R. Electrophoretic migration was from top to bottom. Only one slot is shown here. (B) Densitometric scan of a Fast Green stained $NaDodSO_4$–polyacrylamide cylindrical gel containing about 19 μg of PC-tubulin. Migration was from left to right. The slight deviation from baseline in the region from 0 to 0.5 cm is produced by refraction of light due to the curvature of the gel surface. The sharp spike at the dye front (at 7.8 cm) represents protein (primarily tubulin) that migrates anomalously in this discontinuous gel system (Brewer et al., 1974; our own unpublished results).

volume should increase for a dissociating protein, while no change in peak position should be observed for a noninteracting protein.

Electron Microscopy. Samples for electron microscopy were fixed for 5 min at room temperature by 1:1 dilution with 2% aqueous glutaraldehyde. After the protein concentration was adjusted to 0.2–0.8 mg/mL with PM, samples were applied to carbon-coated collodion grids and negatively stained. A drop of sample was deposited on a grid for 1 min, excess solution was drawn off from the top with filter paper, and a drop of 1% aqueous uranyl acetate was applied for 1 min and drawn off. Grids were allowed to air dry. Samples were observed with a Hitachi HU-11B electron microscope.

Analytical Ultracentrifugation. Centrifugation was performed with the use of a Beckman Model E analytical ultracentrifuge equipped with Rayleigh interference optics and a helium–neon laser light source (Williams, 1972). Sedimentation velocity experiments were conducted at 44 000 rpm and 4.7 °C in cells with double-sector aluminum-filled Epon centerpieces of 12-mm or 30-mm optical path and sapphire windows. At least one blank run using distilled water was performed with each experimental run. Sedimentation coefficients were calculated from plots of the natural logarithm of the square root of the second moment of the interference fringe boundary (Schachman, 1959) vs. time, and corrected to 20 °C and water. Sedimentation equilibrium studies were performed according to the high speed (meniscus depletion) technique of Yphantis (1964) at 4.6 °C, with the use of "external loading" cells of 30-mm optical path (Ansevin et al., 1970) in an An-E rotor. The height of the solution column in each channel was 3.2 mm. Apparent weight-average molecular weights, $M_w(r)$, at each radial position were calculated from the blank-corrected data by a running-fit program similar to those described by Roark & Yphantis (1969) and by Teller (1973). A value of 0.726 mL/g was used for the partial specific volume (calculated according to the method of Cohn & Edsall (1943) from the amino acid composition of bovine brain tubulin presented by Lee et al. (1973)). Samples were run at three different initial concentrations in each run and at two different rotor speeds (different experiments). Sedimentation to equilibrium at different initial concentrations and rotor speeds provides a sensitive test for heterogeneity within a protein sample; alternatively, overlap of plots of molecular weight vs. protein concentration indicates a homogeneous, reversibly dissociating system (Roark & Yphantis, 1969; Teller, 1973). Since the α and β chains of tubulin are of approximately identical molecular weight (Bryan & Wilson, 1971; Lee et al., 1973; Ludueña & Woodward, 1975), the dissociation constant, K_D, is equal to the concentration at which the dimer is half dissociated divided by two. The molar dissociation constant may be approximated by

$$K_D(M) \simeq K_D(g/L) \times 2/M_1$$

where M_1 equals the "monomer" molecular weight of 55 000.

Control Calculations. In order to simulate the curves of $M_w(r)$ vs. concentration that would result from hypothetical noninteracting mixtures, it was assumed that ideality prevails and that the ith component will redistribute itself at sedimentation equilibrium in a manner independent of the presence of other components:

$$C_i(r) = C_{i,0} \exp[\sigma_i (r^2 - r_0^2)/2]$$

where $C_{i,0}$ is the meniscus concentration of the ith species at equilibrium and $\sigma_i = M_i\omega^2(1 - \bar{v}\rho)/RT$. For a given hypothetical mixture, $C_i(r)$ was calculated at many points for each species. At each of the points, the weight-average molecular weight of all species, $M_w(r)$, was calculated, and the total concentration of all species was noted. Rotor speeds, radii, and initial concentrations were chosen to approximate closely those of the actual experiments.

Results

Characterization of the Tubulin Employed. Homogeneity of the PC-tubulin was examined by $NaDodSO_4$–polyacrylamide gel electrophoresis. Figure 1A shows one slot (74 μg of PC-tubulin) of a slab gel stained with Coomassie Brilliant Blue R. In agreement with previous studies (Weingarten et al., 1975; Sloboda et al., 1976; Himes et al., 1976; Herzog & Weber, 1977; Himes et al., 1977; Wehland et al., 1977), no τ or HMW proteins are visible in this preparation. The only contaminants

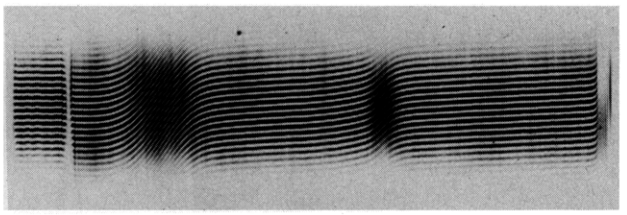

FIGURE 2: Rayleigh interference fringe pattern from a sedimentation velocity experiment on PC-purified tubulin at 2.54 mg/mL. Sedimentation was performed at 44 000 rpm and 4.7 °C in a cell of 30-mm optical path. Sedimentation was from left to right. Two distinct boundaries are visible: the large one on the left moved at approximately 6 S, and the small one to the right of center moved at about 19 S.

present are four bands of apparent molecular weight less than 55 000. Figure 1B is a scan of a cylindrical gel (19 μg of PC-tubulin) stained with Fast Green. The four minor bands (arrows) are evident. Integration of the areas under the five peaks shows that tubulin comprises 96 ± 1.4% of the stained density. If Fast Green stains tubulin and the impurities with equal intensity, then tubulin comprises 96 ± 1.4% of the protein present. The low molecular weight bands appear to stain relatively less intensely with Coomassie Blue; therefore, the degree of purity determined by Fast Green analysis may be an underestimate.

The PC-tubulin preparation displays two boundaries when examined by velocity sedimentation at 4.7 °C, as shown in Figure 2. The first boundary (approximately 6 S, see below) represents tubulin, while the second boundary corresponds to a larger aggregate of tubulin (19 to 20 S), apparently in rapid equilibrium with the material in the 6S boundary.

Sedimentation Equilibrium Studies. The experimental results for three separate equilibrium runs at two speeds and nine different initial protein concentrations are shown in Figure 3. Locally determined values of weight-average molecular weight, $M_w(r)$, have been plotted as a function of concentration. The value of $M_w(r)$ increases from approximately 80 000 to approximately 110 000 over the concentration range studied. Three points can be made about these results. First, the data from all channels and experiments superimpose to a significant degree, as one would expect for a solute that participates in a rapidly reversible association equilibrium (Roark & Yphantis, 1969; Teller, 1973). Second, the weight-average molecular weight reaches a value halfway between that of monomer and dimer at a concentration near 0.02 mg/mL. Third, at concentrations above about 0.4 mg/mL, the points deviate upward from the hyperbola predicted for dimerization of two 55 000 molecular weight monomers. This deviation almost certainly results from the formation of higher aggregates of tubulin, most probably corresponding to the 19S boundary seen in velocity sedimentation.

Because of the presence of the higher aggregates, which are of unknown molecular weight, precise curve-fitting to give a monomer–dimer association constant is not possible. However, from the points below 0.4 mg/mL, one may estimate a value of K_D of approximately 8×10^{-7} M, corresponding to a free energy of dissociation of 7.8 kcal/mol, at 4.6 °C in PM buffer.

Control experiments were performed to measure the extent to which the PC-purified dimer remained competent to bind colchicine and to polymerize into microtubules during the long time course (up to 26 h) of the sedimentation equilibrium experiments. [^{3}H]Colchicine binding assays were performed at the beginning and end of each run (on aliquots of the most concentrated solution employed for the run) and the results are summarized in Table I. In all cases the protein retained at

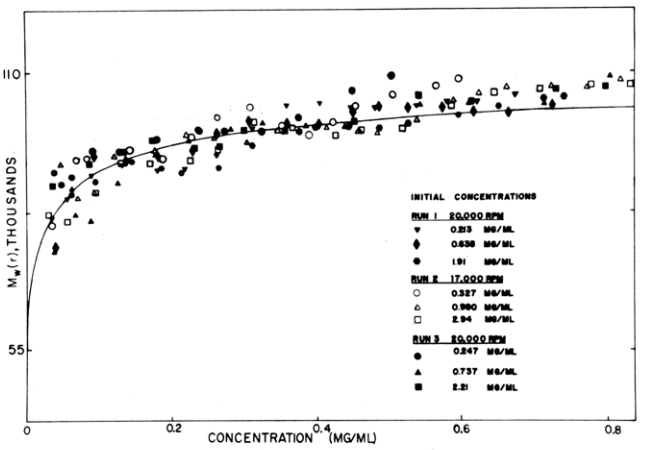

FIGURE 3: Weight average molecular weight of PC-tubulin as a function of its concentration in PM buffer (0.1 M Pipes–KOH (pH 6.9), 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM GTP, 2 mM DTE, ionic strength 0.236). Initial concentrations and rotor speeds are as indicated. Runs 1 and 2 were from one preparation of tubulin; run 3 was from another. Centrifugation was performed at 4.6 °C, and interference patterns were recorded between 15 and 30 h after the start of each run. Minimum times to equilibrium were established from the relation of Van Holde & Baldwin (1958) for $T = 5$ °C and $D_{5°,w} = 3.4 \pm 0.4 \times 10^{-7}$ cm^2/s (Steven A. Berkowitz, personal communication).

TABLE I: Colchicine Binding Assays Accompanying Sedimentation Equilibrium Experiments.

run and symbols	time after start of experiment (h)	[C]/[T] tubulin peak[a]	% retention of colchicine-binding[b] activity
1	8	0.826 ± 0.080	
▼ ♦ ●	32	0.583 ± 0.090	70.6
2	3.5	0.896± 0.040	
○ △ □	25.5	0.629	70.2
3	3.5	0.898 ± 0.026	
● ▲ ■	25.5	0.654 ± 0.034	72.8

[a] Molar ratios are averages of values obtained using A_{280} and the Bradford assay to calculate the concentration of tubulin. Samples were maintained at 4 °C between measurements. Values are uncorrected for the loss in CBA during the 1.5-h incubation at 37 °C. [b] Percent retention of CBA is expressed as percent of initial measurement.

least 70% of its initial colchicine-binding activity. Furthermore, microtubules could be polymerized from a sample of tubulin (2.2 mg/mL) maintained at 4 °C for 6 h (Figure 4A,B) and for 26 h (Figure 4C) after the start of sedimentation equilibrium run 3. At both times, substantial turbidity developed after incubation of the tubulin sample for 45 min at 37 °C and this turbidity increase was cold reversible. A colchicine-treated control is shown in Figure 4D. No microtubules are present, although numerous globular particles, which are probably aggregates of tubulin, are evident. The slow decay of colchicine binding observed in these experiments and the polymerization competence of 26-h-old tubulin probably resulted from two factors: the protein was maintained at 4 °C (cf. Pfeffer et al., 1976) between measurements since a similar temperature prevailed in the centrifuge cells, and the experimental buffer contained 2 mM DTE, which would be expected to protect sulfhydryl groups essential for polymerization and those involved in colchicine binding (Kuriyama & Sakai, 1974). We conclude from these controls that a substantial fraction of protein remained in its native state during the sedimentation equilibrium experiments.

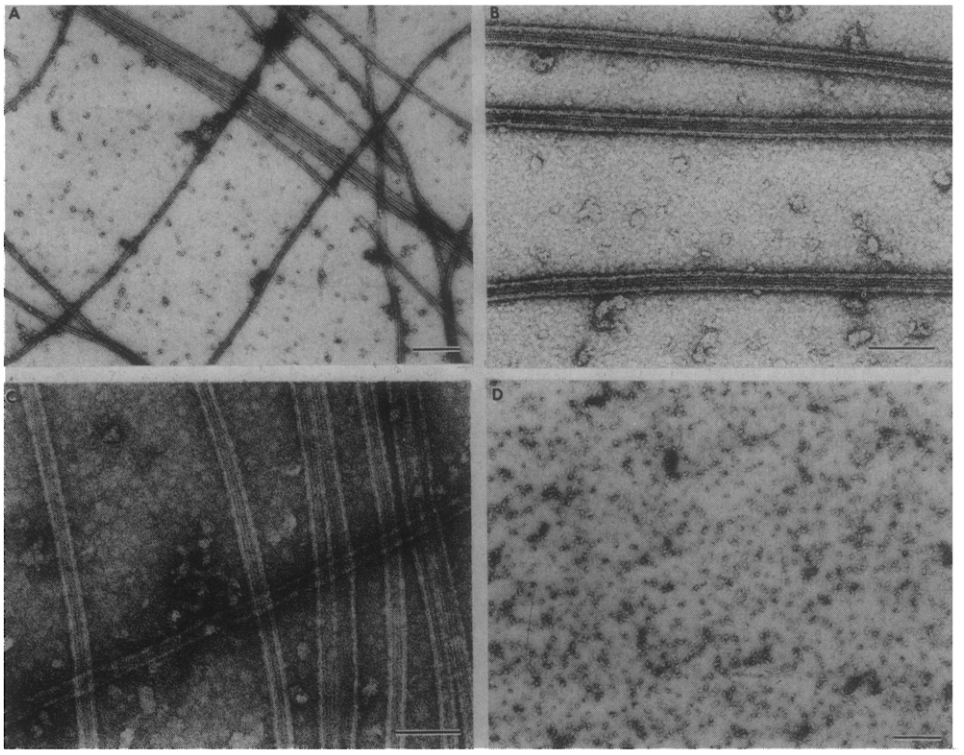

FIGURE 4: Electron micrographs of negatively stained samples (for preparation see Materials and Methods). (A) PC-tubulin polymerized in PM buffer 6 h after the start of sedimentation equilibrium run 3 (see Figure 3). Polymerization was for 45 min at 37 °C. Bar = 0.3 μm. (B) Same as in A, but a different field. Bar = 0.1 μm. (C) PC-tubulin of A, polymerized 26 h after start of equilibrium run 3. Bar = 0.1 μm. (D) Tubulin incubated with 1 mM colchicine for 1.5 h at 37 °C, 6 h after start of run 3. Bar = 0.3 μm. Grids of tubulin maintained at 4 °C for 6 h were also examined. No tubules were observed.

A series of control computations was carried out to ascertain that the observed curves of weight-average molecular weight were not the result of phenomena other than the rapidly reversible dissociation of the tubulin dimer. A first such possibility, suggested by the 30% loss of colchicine binding capacity during the time of the experiment, is that the system is a nonequilibrating mixture of dimer and as much as 30% monomer, perhaps in a denatured state. A second such possibility, suggested by the gel electrophoresis results, is that the system consists of nondissociating tubulin dimer mixed with approximately 5% of contaminating proteins of lower molecular weight. Both possibilities were calculated as described in Materials and Methods. (Since the size of the 19S aggregate is not known, the simulations were confined to dimeric tubulin and its subunits.) Figure 5A shows the situation that would obtain if 10% of the tubulin were present as nonassociating monomer, and 90% as nondissociating dimers. Figure 5B shows the situation if tubulin were a nondissociating dimer contaminated with 5% of a protein of molecular weight 25 000. In each of these two cases it is easily seen that the calculated amount of nonoverlap of the curves of $M_w(r)$ vs. concentration greatly exceeds the amount observed experimentally (Figure 3). Thus, neither the hypothetical presence of noninteracting monomer (α- or β-tubulin) in amounts comparable to the amount of non-colchicine-binding tubulin, nor the hypothetical presence of noninteracting proteins in the amounts, and of the molecular weight, of the observed impurities can account for the observed decrease in $M_w(r)$ with decreasing concentration.

Concentration Dependence of Sedimentation Velocity. As shown in Figure 2, PC-tubulin exhibited two components when sedimented in PM buffer at 4.7 °C and 44 000 rpm. The concentration dependence of the sedimentation coefficient of the slow boundary is shown in Figure 6. At low protein concentrations (<0.5 mg/mL) the sedimentation coefficient of the slow boundary decreases, in a manner consistent with the presence of a reversible equilibrium between the tubulin dimer and its monomers.

Gel Filtration Chromatography. Phosphocellulose-purified tubulin was chromatographed at 4 °C on a composite column of Bio-Gel P-150 and Sephadex G-25 at a wide range of initial concentrations. The composite column was designed to detect the dissociation of the tubulin dimer into its subunits. The molecular weight exclusion limit calculated from the calibration data was approximately 110 000. Therefore, any peak elution volume greater than the void volume would indicate the presence of a mixture of dimer and dissociated monomer.

Three representative elution profiles are shown in Figure 7A. It is apparent that peak elution volume increases as the concentration of the tubulin loaded on the column decreases. The data from a number of such experiments are summarized in Figure 7B. Peak elution volume is plotted as a function of column load concentration. At the lowest concentrations loaded, the peak eluted at the same volume as BSA, indicating that substantial dissociated monomer is present. Note that the elution position for BSA (▲), a nonassociating protein, does not change significantly despite a tenfold change in column load concentration.

Reconcentration Experiments. To demonstrate directly the reversible nature of the dissociation of the tubulin dimer, a sample of reconcentrated tubulin was examined by sedimentation velocity ultracentrifugation and gel filtration chromatography. Tubulin (8 mg/mL) was diluted to 0.20 mg/mL with PM buffer, incubated on ice for 1 h, reconcentrated by pressure ultrafiltration, degassed gently, and centrifuged at 5000*g* for 10 min. The total time required for reconcentration and subsequent preparation was 1.5 h. A 2.0-mL sample (1.19 mg/mL) was then chromatographed on the composite gel

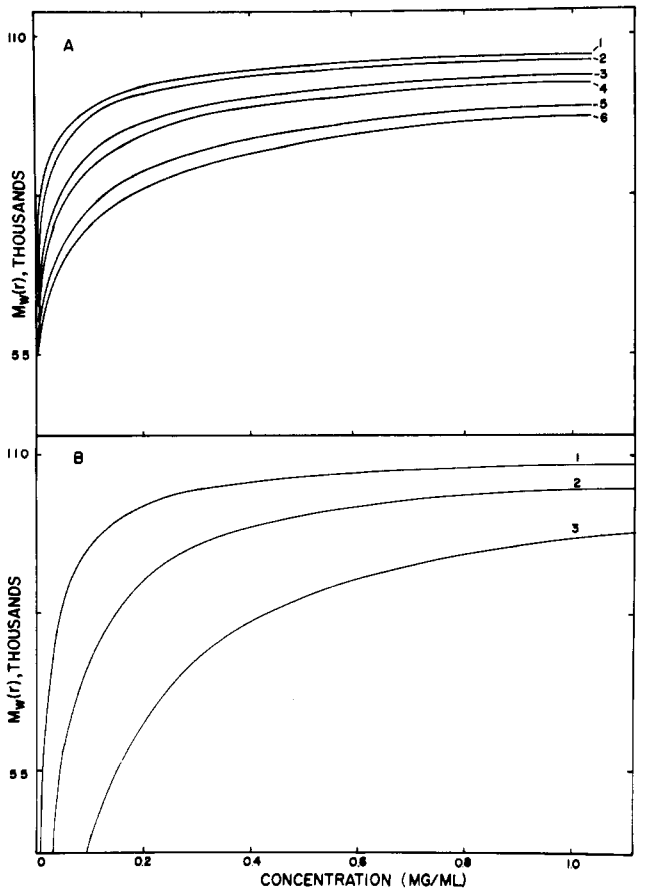

FIGURE 5: Simulation of the effects of heterogeneity, for comparison to Figure 3. Computations were performed as described in Materials and Methods. The temperature for all calculations was 5 °C. (A) The distributions of $M_w(r)$ that would be observed for a two-component, noninteracting system composed of 10% nonassociating monomer and 90% nondissociating dimer. Initial concentrations: (1 and 2) 0.334 mg/mL; (3 and 4) 1.00 mg/mL; (5 and 6) 3.01 mg/mL. Rotor speeds: odd numbers = 17 000 rpm; even numbers = 20 000 rpm. (B) The concentration dependence of $M_w(r)$ that would be observed for a system containing 95% nondissociating tubulin dimer and 5% nonequilibrating protein of mol wt 25 000. Initial concentrations: (1) 0.334 mg/mL; (2) 1.00 mg/mL; (3) 3.01 mg/mL. Rotor speed = 20 000 rpm.

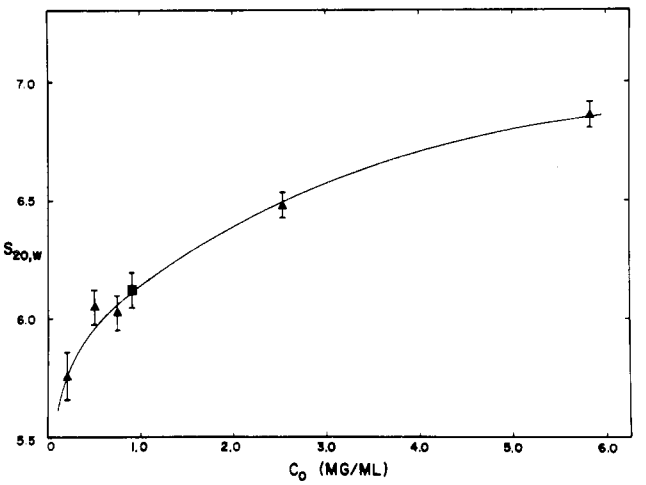

FIGURE 6: Concentration dependence of the sedimentation coefficient of PC-tubulin. Protein stored in liquid nitrogen was thawed rapidly, centrifuged at 5000*g* for 10 min, and eluted from a column of Sephadex G-25 (0.9 cm × 25 cm), equilibrated with PM buffer. Sedimentation was performed at 44 000 rpm and 4.7 °C (▲). The square point (■) represents a sample reconcentrated from 0.2 to 0.910 mg/mL, as described in Results. Error bars represent estimated maximum limits of experimental uncertainty. The curve is drawn to indicate the trend of the data.

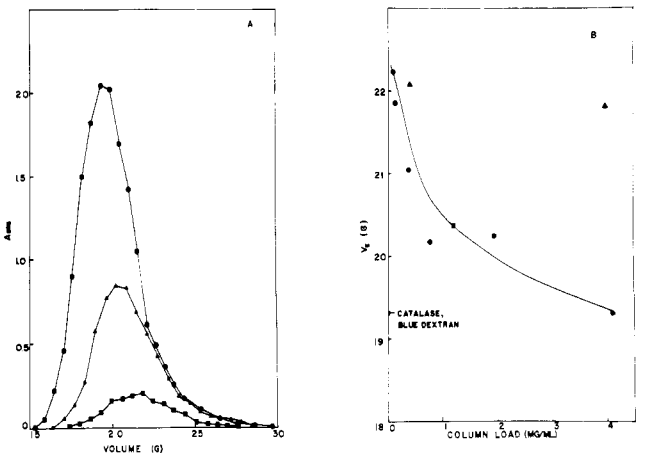

FIGURE 7: Bio-Gel P-150 chromatography. PC-tubulin at various initial concentrations was chromatographed on a 0.9 × 65 cm column of Bio-Gel P-150 overlayed with 18 cm of Sephadex G-25. The column was equilibrated and developed with PM buffer. Fraction weights were recorded and protein concentrations were monitored by the method of Bradford (1976). A modified Bradford assay (0.5 mL of sample + 5.0 mL of reagent) was used to determine the column profiles at low loading concentrations (0.076 mg/mL and 0.122 mg/mL experiments). (A) Representative elution profiles for three different column-load concentrations: 4.11 mg/mL (●); 1.94 mg/mL (▲); 0.122 mg/mL (■). (B) Tubulin peak elution volume (expressed in grams) as a function of the concentration of the column load (●). The square point (■) represents tubulin reconcentrated to 1.19 mg/mL as in Figure 6, with the omission of Sephadex G-25 chromatography. The triangular points (▲) represent the elution position of bovine serum albumin. The curve is drawn to indicate the trend of the data.

filtration column, while another sample was chromatographed on G-25 to prepare it for velocity sedimentation (concentration after G-25 chromatography was 0.910 mg/mL). The results are represented by the square symbols in Figures 6 and 7B. The sedimentation coefficient and elution volume of the reconcentrated samples are seen to depend only on the concentration at which the experiments were performed and not on the prior history (dilution to 0.20 mg/mL) of the sample. For example, reconcentrated tubulin at 0.910 mg/mL sedimented at 6.12 S, in contrast to the 5.76 S observed for a sample at 0.20 mg/mL. These findings provide direct evidence of the reversibility of the dissociation of the tubulin dimer and indicate that the process is relatively rapid, with equilibration occurring within at most 1.5 h.

The relatively rapid dissociation of the tubulin dimer produced by dilution and incubation on ice was verified by velocity analysis of a sample of the same PC-tubulin preparation between 1 and 3 h after dilution to 0.19 mg/mL. The ultracentrifuge rotor attained speed 45 min after dilution, and the boundary position was measured in the ensuing 2 h. The observed sedimentation coefficient was 5.68 ± 0.10 S. This result demonstrates that prior to reconcentration the sample had in fact already dissociated and approached a new state of equilibrium.

The effect of the reconcentration procedure on the viability of the tubulin sample was examined by determination of the first-order decay parameters for colchicine binding. In Figure 8 molar ratios of colchicine to tubulin (●) and the natural logarithm of the percent remaining activity (▲) are plotted as a function of storage time at 4 °C. These values are uncorrected for the loss in colchicine-binding activity during the incubation for 1.5 h at 37 °C. The *y* intercept of the molar ratio line indicates an initial binding capacity of 0.780. The first-order decay constant obtained from the slope is 0.0224 h^{-1} and the half-time for decay at 4 °C is approximately 31 h. These

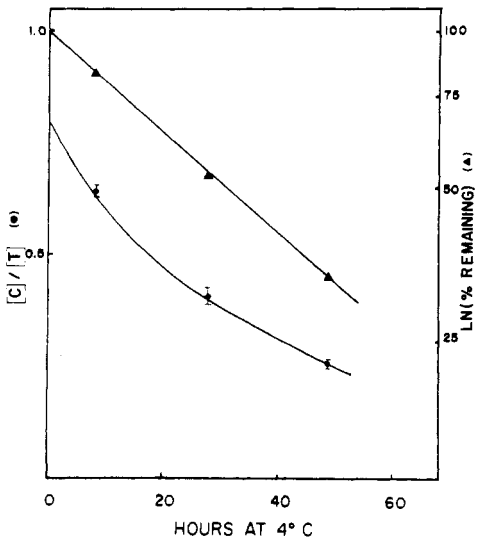

FIGURE 8: First-order decay of colchicine-binding activity of reconcentrated tubulin. Colchicine binding assays were performed on samples of reconcentrated tubulin (1.19 mg/mL) at three different times using the G-25 chromatography method. Molar ratios of colchicine to tubulin (●) and the natural logarithm of the percent remaining activity (▲) are plotted as a function of storage time at 4 °C. These values are uncorrected for the loss of colchicine-binding activity during the 1.5-h incubation at 37 °C.

results indicate that at least 80% of the protein retained its ability to bind colchicine, and therefore that the reconcentration process had little deleterious effect on the sample.

Discussion

The data presented above demonstrate that the $\alpha\beta$ dimer of bovine brain tubulin undergoes a rapidly reversible, concentration-dependent dissociation into its subunits under near-physiological conditions of pH and ionic strength. An alternative statement would be that the tubulin dimer is in labile equilibrium with its monomers under these conditions. The dissociation constant is approximately 8×10^{-7} M in PM buffer at 4.6 °C. Several items of evidence leading to this conclusion merit discussion.

The nontubulin proteins observed as components of the PC-tubulin preparation appear to be similar to the LMW-MAPs described by Berkowitz et al. (1977), and to the proteins of molecular weight less than that of tubulin reported by Herzog & Weber (1977). The estimate of their amount is rendered somewhat difficult by the presence of the artifactual sharp spike (Figure 1B) at the dye front. Nevertheless, they can comprise no more than 5% of the total protein present. The absence of proteins of molecular weight higher than that of tubulin is clear from Figure 1A and agrees with previous studies (Weingarten et al., 1975; Sloboda et al., 1976; Himes et al., 1976; Herzog & Weber, 1977; Himes et al., 1977; Wehland et al., 1977).

The presence of associated species larger than the tubulin dimer is clear from Figure 2. The fast boundary which represents these species has an apparent sedimentation coefficient ($s_{20,w}$) of 19.5 ± 0.5 S. The size of this boundary decreases with decreasing protein concentration, and the concentration gradient between it and the slow boundary never reaches zero. These facts suggest strongly that the 19S boundary represents a species in rapidly reversible equilibrium with the tubulin monomer–dimer system (i.e., with the slow boundary), and that it is similar to the Mg^{2+}-induced polymer seen by Frigon & Timasheff (1975) in experiments with calf brain tubulin in a different buffer system. If so, it would consist of 26 tubulin dimers and have a molecular weight approximately 2.86 million. This aggregate must be present, although not directly visible, in the sedimentation equilibrium and gel filtration experiments. In the former experiments, it would be effectively "pelleted" at the base of the ultracentrifuge cell and thus will contribute relatively little effect in the region near the meniscus where the monomer–dimer equilibrium is measured. In the gel filtration experiments, the aggregate will emerge at the front of the peak but will not be resolved from it because its size exceeds, and the size of tubulin approaches, the exclusion limit of the gel (approximately 150 000 daltons). The contribution of the large aggregate to the gel filtration results will make the tubulin dimer appear, at a given concentration, to be somewhat less dissociated than it truly is. In any case, the 19S material is present in negligible amount at the very low loading concentrations which provide substantial evidence for dissociation of the dimer into its subunits.

The relatively (cf. Wilson et al., 1974; Sherline et al., 1975) slow decay of colchicine-binding activity and of the capacity to polymerize to form microtubules suggest strongly that the monomer–dimer equilibrium is a property of native tubulin rather than an artifact arising from its degradation. The apparent homogeneity of the protein with regard to its dissociability, reflected in the overlap of curves in Figure 3, gives further strength to this interpretation, as does the readjustment of the monomer–dimer equilibrium in response to reconcentration of previously diluted (and therefore dissociated) tubulin. Although the particular solution used for the reconcentration experiments was not examined by velocity sedimentation or by gel filtration when dilute, velocity analysis of a sample of the same preparation of PC-tubulin at 0.19 mg/mL under identical experimental conditions indicated that the elapsed time of approximately 1 h in the dilute state was long enough to allow dissociation equilibrium to be closely approached.

The colchicine-binding assay has been employed as a quantitative indicator of the native state of the tubulin dimer in our studies. Recently, Barton (1978) has concluded that polymerization and colchicine-binding assays measure independent properties of tubulin. However, since those studies were performed on once-cycled porcine brain microtubule protein, the results reflect properties of a multicomponent system (the tubulin dimer and MAPs) and not characteristics of tubulin per se. In this regard, Wiche et al. (1977) have concluded that the divergence between colchicine-binding and polymerization decay rates of rat C_6 glial cell microtubule protein results from the rapid decay of an endogenous factor, perhaps τ, required for polymerization but not for colchicine binding. Therefore, we believe that the colchicine-binding assay is a legitimate tool for determining the stability of the tubulin dimer in pure solution and that the decay rate of colchicine-binding activity probably correlates with the polymerization competence of the tubulin dimer itself.

Recently, information concerning the conditions for microtubule assembly in vitro has appeared. Our observation that microtubules will assemble in vitro from PC-tubulin devoid of HMW-MAPs or τ agrees with a growing number of reports (Lee & Timasheff, 1975; Erickson & Voter, 1976; Herzog & Weber, 1977; Himes et al., 1977; Lee & Timasheff, 1977; Wehland et al., 1977) that these accessory proteins are not an absolute requirement for microtubule polymerization. In our hands, the assembly of PC-tubulin at concentrations above 2 mg/mL in PM buffer does not require high levels of Mg^{2+} (Herzog & Weber, 1977; Lee & Timasheff, 1975), glycerol (Himes et al., 1977; Wehland et al., 1977), Mg^{2+} and glycerol (Lee & Timasheff, 1975; Erickson & Voter, 1976; Lee & Timasheff, 1977), or Me_2SO (Himes et al., 1976, 1977). How-

ever, our PC-tubulin was purified by a protocol involving glycerol (Berkowitz et al., 1977) and presumably has some glycerol tightly bound to it (Detrich et al., 1976). It is possible that the low molecular weight polypeptides ($M_r \simeq 25\,000$–$30\,000$) present in our PC-tubulin preparations possess assembly-stimulating activity. In this regard Vallee & Borisy (1977) have concluded that a low molecular weight HMW fragment (M_r = 31 000–35 000) produced by brief trypsin digestion of in vitro assembled cytoplasmic microtubules may be the essential portion of the HMW proteins required for microtubule assembly.

The in vivo significance of the results reported here is unclear. However, dissociation of the dimer may be part of the mechanisms for control of microtubule assembly. The results do show that a given tubulin dimer is not a permanently linked unit. Rather, it is free to interchange subunits with other dimers, both in vitro and in vivo.

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