David-Pfeuty, T., Erickson, H. P., & Pantaloni, D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5372–5376.
David-Pfeuty, T., Simon, C., & Pantaloni, D. (1979) *J. Biol. Chem. 254*, 11696–11702.
Geahlen, R. L., & Haley, B. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4375–4377.
Geahlen, R. L., & Haley, B. E. (1979) *J. Biol. Chem. 254*, 11982–11987.
Huitorel, P. (1979) M.S. Thesis, Orsay.
Jacobs, M., & Huitorel, P. (1979) *Eur. J. Biochem. 99*, 613–622.
Jacobs, M., Smith, H., & Taylor, E. W. (1974) *J. Mol. Biol. 89*, 455–468.
Karr, T. L., & Purich, D. L. (1978) *Biochem. Biophys. Res. Commun. 84*, 957–961.
Karr, T. L., Podrasky, A. E., & Purich, D. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5475–5479.
Kobayashi, T. (1975) *J. Biochem. (Tokyo) 77*, 1193–1197.
Lee, J. C., & Timasheff, S. N. (1975) *Biochemistry 14*, 5183–5187.
Lowry, O., Rosebrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.
MacNeal, R. K., & Purich, D. L. (1978a) *J. Biol. Chem. 253*, 4683–4687.
MacNeal, R. L., & Purich, D. L. (1978b) *Arch. Biochem. Biophys. 191*, 233–243.
Margolis, R. L., & Wilson, L. (1978) *Cell (Cambridge, Mass). 13*, 1–8.
Murphy, D. B., Vallee, R. B., & Borisy, G. G. (1977) *Biochemistry 16*, 2598–2605.
Pardee, J., & Spudich, J. A. (1979) *Cold Spring Harbor Symp., Abstr.*, 27.
Penningroth, S. M., & Kirshner, M. W. (1977) *J. Mol. Biol. 115*, 643–673.
Schiff, P. B., Fant, J., & Hortwitz, S. B. (1979) *Nature (London) 277*, 665–667.
Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.
Wegner, A. (1976) *J. Mol. Biol. 108*, 139–150.
Weisenberg, R. C. (1980) *J. Mol. Biol. 139*, 660–677.
Weisenberg, R. C., & Deery, W. J. (1976) *Nature (London) 263*, 792.
Weisenberg, R. C., Deery, W. J., & Dickinson, P. J. (1976) *Biochemistry 15*, 4248–4254.
Wiegand, G., & Kaleja, R. (1976) *Eur. J. Biochem. 65*, 473–479.
Zackroff, R. V., Weisenberg, R. C., & Deery, W. J. (1980) *J. Mol. Biol. 139*, 641–677.

Conformation and Assembly Characteristics of Tubulin and Microtubule Protein from Bovine Brain†

David C. Clark, Stephen R. Martin, and Peter M. Bayley*

ABSTRACT: The conformational requirements for the efficient assembly of bovine brain tubulin into microtubules have been investigated by using near-UV circular dichroism. Microtubule protein was prepared by the assembly–disassembly method of Shelanski et al. [Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768]. Tubulin dimer, isolated from this multiprotein complex by phosphocellulose ion-exchange chromatography in the presence and absence of Mg^{2+}, was compared with tubulin dimer (WT dimer) prepared by the method of Weisenberg & Timasheff [Weisenberg, R. C., & Timasheff, S. N. (1970) *Biochemistry 9*, 4110–4116]. The tubulin from both dimer preparations showed identical electrophoretic patterns in which high molecular weight protein was undetectable. However, reproducible and significant differences were found in the near-UV CD spectra. Phosphocellulose-treated tubulin resembles the original microtubule protein more closely than does WT dimer, although this latter material has been widely accepted as being representative of the native protein. The phosphocellulose-treated tubulin and WT dimer are not readily interconvertible by simple physical or chemical treatments. The assembly capability of the various tubulin dimer preparations was compared by measuring the enhancement by tubulin dimer of assembly of ring fraction (isolated from microtubule protein by gel filtration on Sepharose 6B). Again phosphocellulose-treated tubulin is found to behave more like native microtubule protein than does WT dimer.

Microtubules, organelles found in all eukaryotic cells, have been the subject of considerable interest in recent years and reviewed recently (Snyder & McIntosh, 1976; Jacobs & Cavalier-Smith, 1977; Kirschner, 1978; Dustin, 1978). Some of the cellular functions which involve microtubules (e.g., mitosis) are dependent upon the ordered assembly and disassembly of these structures. Also, the binding of certain alkaloid drugs (e.g., colchicine; Wilson et al., 1974) and the variation in a number of external physical parameters (e.g., low temperature and high pressure) have been shown to dissociate microtubules reversibly in eukaryotic cells, leading to disruption of a variety of cellular functions (Olmsted & Borisy, 1973) and providing evidence for the involvement of microtubules in chromosome movements in cell division, intracellular organization and transport, development and maintenance of cell shape, cellular motility, and sensory transduction.

A number of different methods of isolation have been developed for the proteins comprising microtubules, including the essentially classical biochemical isolation via ammonium sulfate precipitation and column chromatography to yield

† From the Biophysics Division, National Institute for Medical Research, Mill Hill, London, NW7 1AA England. *Received August 5, 1980.*

0006-2960/81/0420-1924$01.25/0

purified tubulin dimer (WT dimer) as a single homogeneous protein (Weisenberg & Timasheff, 1970; Lee et al., 1973). By contrast, the procedure of Shelanski et al. (1973) uses multiple cycles of assembly and disassembly, subjecting the material only to temperature changes in the presence of glycerol and guanine nucleotides. We use the terminology of "microtubule protein" (MT protein) to indicate material prepared by this procedure and which contains a number of microtubule-associated proteins (MAPs) such as HMW1, HMW2 (M_r 300000 and 280000; Herzog & Weber, 1978), and the group of τ proteins (M_r 58000–66000; Witman et al., 1976) in addition to the major component, the tubulin $\alpha\beta$ heterodimer, M_r 110000 (Frigon & Timasheff, 1975).

The ability to perform reversible assembly–disassembly of microtubules in vitro (Weisenberg, 1972; Shelanski et al., 1973) has led to wide-ranging studies of factors which affect the efficiency of this process. These include divalent ions and guanine nucleotides and the physical conditions (temperature, pH, buffer ion type and concentration, and the presence of sucrose, glycerol, poly(ethylene glycol), or dimethyl sulfoxide in the solvent). Two related factors are of particular importance, namely the concentration of tubulin itself and the presence or absence of MAPs, the microtubule-associated proteins. Whereas tubulin dimer may be induced to undergo assembly in the absence of MAPs, the presence of MAPs reduces the critical concentration for polymerization to ~0.1 mg/mL, and this has led many workers to assume a specific function in vivo for these proteins, a postulate supported by electron microscopy (Amos, 1977) and immunofluorescence studies (Sheterline, 1978).

Cold dissociated microtubule protein may be fractionated on the basis of molecular weight to yield tubulin dimer and an oligomeric complex (containing tubulin dimer plus MAPs) in the form of a "ring" fraction as identified by electron microscopy (Erickson, 1974). This complex is functional in the sense that it too may be assembled at 37 °C in the presence of GTP, and, compared to MT protein, it represents a fraction somewhat enriched in MAPs (Vallee & Borisy, 1978).

Several workers have investigated the conformational properties of WT dimer by using circular dichroism (CD) spectroscopy (Ventilla et al., 1972; Lee & Timasheff, 1977; Lee et al., 1978). These studies have shown that tubulin has a typical globular conformation containing approximately 22–26% α helix and 30–47% β structure. The conformation of tubulin can be protected against destabilization at increased temperatures by the presence of nucleotides and antimitotic drugs (Lee et al., 1978). The near-UV CD of the aromatic amino acid region shows a progressive loosening of the structure with increasing pH and major unfolding at pH 9. Lee & Timasheff (1977) have also shown that those solution variables which promote assembly of WT dimer in vitro have little effect on the near-UV CD.

The purpose of this work is to examine the conformational requirements for efficient assembly of tubulin prepared by different methods. It appears likely that an essential step in the assembly process of microtubule protein involves the association of tubulin and MAPs in the formation of a multiprotein complex. We have therefore investigated the conformational relationship between Shelanski-prepared microtubule protein and the tubulin dimer derived from it by phosphocellulose ion-exchange chromatography in the absence and presence of added Mg^{2+} (Williams & Detrich, 1979). We have also prepared WT dimer directly from brain tissue by the fractionation procedure of Weisenberg & Timasheff (1970). We have compared all these preparations by characterization studies employing absorption spectroscopy, circular dichroism spectroscopy, and $NaDodSO_4$–polyacrylamide gel electrophoresis. We have also investigated the assembly capability of each preparation by a turbidimetric assembly assay based on the assembly properties of the oligomeric ring fraction isolated from microtubule protein by gel chromatography (Sloboda et al., 1976).

Materials and Methods

Bovine brains were obtained from British Beef Ltd., Watford, England. GTP,[1] Type II-S, and ATP were purchased from Sigma Chemical Co. Phosphocellulose (Whatman P11) was swollen in water and treated before use by washing with 50% ethanol, 0.5 M NaOH, water, 0.5 M HCl, and finally water as described by Weingarten et al. (1975). DEAE-Sephadex, Sephadex G-25, and Sepharose 6B were all obtained from Pharmacia. Eriochrome Black T (Eastman Kodak Co.) was recrystallized from ethanol as described by Diehl & Lindstrom (1959). Methylenebis(acrylamide) (British Drug Houses, electrophoresis grade) was recrystallized from acetone. All other chemicals were "Analar" grade and used without further purification.

Protein Preparation. Microtubule protein complex (MT-protein complex) was prepared from fresh bovine brain by two cycles of assembly–disassembly similar to the method described by Shelanski et al. (1973) but with the following modifications. Homogenization (2 × 30 s MSE Atomix blender) of washed grey matter was performed in purification buffer (0.1 M Mes, 1 mM EGTA, 0.5 mM $MgCl_2$, and 5 mM β-mercaptoethanol, pH 6.5) containing 1 mM ATP in the ratio 0.5 mL of buffer/g of brain tissue. The 100000*g* supernatant was assembled by addition of an equal volume of purification buffer containing 0.5 mM GTP (included in all purification buffers from this stage) plus 8 M glycerol and incubated for 30 min at 37 °C. The final yield of microtubules could be increased by ~30% by resuspending the pelleted brain tissue from the first homogenate in purification buffer containing 1 mM ATP using the blendor (one 30-s burst) and assembling the 100000*g* supernatant as before. The microtubules formed were pelleted, resuspended, and disassembled by incubation on ice, and the solutions were combined and clarified by centrifugation (100000*g*, 30 min, 4 °C). The purification involved one further cycle of assembly, and the harvested microtubules were resuspended in 0.1 M Mes, 0.1 mM EGTA, and 0.5 mM $MgCl_2$, pH 6.5 (MEM100 buffer) containing 6.5 M glycerol and disassembled at 4 °C. The resulting MT-protein complex solution was stored at −70 °C and used within 6 weeks of preparation.

Before each experiment a suitable aliquot of stored MT-protein complex was removed from storage, thawed, diluted to 4 M in glycerol, and made 0.5 mM in GTP by the addition of MEM100 buffer containing the appropriate amount of GTP. This solution was incubated at 37 °C for 30 min, and the microtubules were harvested by centrifugation (100000*g*, 45 min, 37 °C). The pellets were washed with MEM100 buffer at 37 °C to remove residual glycerol and GTP, resuspended in the required volume of MEM100 buffer, and disassembled by chilling on ice for 30 min. Finally, the solution was clarified by centrifugation (100000*g*, 20 min, 4 °C). This solution thus contained solubilized MT-protein complex gen-

[1] Abbreviations used: GTP, guanosine triphosphate; ATP, adenosine triphosphate; DEAE, diethylaminoethyl; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PC, phosphocellulose; $NaDodSO_4$, sodium dodecyl sulfate.

erally at 6–9 mg/mL, which had been taken through three cycles of assembly.

Weisenberg tubulin (WT dimer) was prepared by the method of Weisenberg & Timasheff (1970). The $MgCl_2$ precipitation step was omitted from the scheme. Thus the eluate from the Sephadex G-25 column was dialyzed for 24 h against 0.01 M phosphate, 5 mM $MgCl_2$, and 10^{-4} M GTP, pH 7.0 (PMG buffer), containing 1 M sucrose and stored at −70 °C. WT dimer was used within 1 week of preparation.

Before each experiment a suitable aliquot of WT dimer was removed from storage, thawed, dialyzed against PMG buffer or a reassembly buffer (0.01 M Mes, 1 mM EGTA, 16 mM $MgCl_2$, 10^{-4} M GTP, and 3.4 M glycerol, pH 7.0), and clarified by centrifugation (100000*g*, 20 min, 4 °C). Generally CD spectra were recorded for these samples directly, but, on occasions, for verification of the integrity of the sample, WT dimer was taken through a cycle of assembly. This was achieved by incubating the sample in the reassembly buffer at 37 °C for 30 min, followed by centrifugation (100000*g*, 40 min, 37 °C). The harvested microtubules were resuspended in PMG buffer and disassembled by incubation on ice for 20 min. The solution was then clarified by centrifugation (100000*g*, 20 min, 4° C) and the CD spectrum recorded immediately.

Chromatography of Microtubule Protein. MAP-free tubulin dimer was prepared from MT-protein complex by chromatography on phosphocellulose. For these experiments the precycled column material (Weingarten et al., 1975) was equilibrated and then run in MEM25 buffer (25 mM Mes, 0.5 mM $MgCl_2$, and 0.1 mM EGTA, pH 6.5) at 4 °C. MT-protein complex was chromatographed routinely at 1 mg of protein/mL of phosphocellulose, and tubulin dimer was eluted with the void volume. Dimer prepared in this manner is termed PCT dimer.

Tubulin dimer was also prepared by chromatography on phosphocellulose that had been presaturated with Mg^{2+} as suggested by Williams & Detrich (1979). Presaturation was achieved by equilibration of normally precycled phosphocellulose with MEM25 buffer containing 100 mM $MgCl_2$. The Mg-saturated phosphocellulose was exhaustively washed with MEM25 buffer to remove unbound magnesium, and the column was poured and then run in the same buffer at 4 °C. Electrophoresis showed initially that chromatography on columns presaturated with Mg^{2+} was less efficient at removing MAPs than normal phosphocellulose treatment. However, resolution was improved by doubling the bed volume of presaturated columns; thus, chromatography was performed in the ratio of 0.5 mg of MT-protein complex/mL of magnesium-presaturated phosphocellulose. Tubulin dimer prepared in this manner is referred to as MgPCT dimer.

Mg^{2+} assays carried out as described below showed that preparations of MgPCT dimer contained Mg^{2+} at the same concentration as that of the running buffer (i.e., 0.5 mM). On the other hand, preparations of PCT dimer contained <20 μM Mg^{2+}.

Higher aggregates, referred to as rings were separated from tubulin dimer by chromatography on Sepharose 6B using MT-protein complex solutions of concentrations between 4 and 8 mg/mL in MEM100 buffer. MT-protein complex (3–4 mL) was applied to a 30 × 2.5 cm column of Sepharose 6B equilibrated with MEM100 containing 4 M glycerol. This column was run at ~60 mL/h at 25 °C, and 1.5-mL fractions were collected. This method, except for the addition of glycerol, is essentially the same as that described by Erickson (1974) and Kirschner & Williams (1974).

Circular Dichroism. CD spectra were recorded on a Jasco J41C spectropolarimeter equipped with a Model J-DPY Data Processor. Digitized spectra collected by the processor were transferred in a Datalab transient recorder DL901 to a Varian 620L-100 laboratory computer and stored on magnetic tape cassettes. After transfer to the main HP3000 computer, spectra were processed for base line subtraction, normalization, and smoothing according to the methods of Savitsky & Golay (1964) using graphics display (Tektronix 4010) and output using a Calcomp 565.

Spectra were routinely recorded from 340 to 250 and 260 to 200 nm at a sensitivity of 50 × 10^{-2} mdeg/cm with an instrumental time constant of 16 s. Fused silica cells of path length 0.1, 1, 2, and 10 mm were used as appropriate. The spectra recorded in this article are the average of multiple scans recorded from several samples from at least two protein preparations. Spectra were recorded at 15 °C unless otherwise specified. All spectra are presented as molar circular dichroism $\Delta\epsilon$, based upon a mean residue weight of 110. Molar ellipticity may be obtained from

$$[\Theta]_{MRW} = 3300\Delta\epsilon$$

Assembly. Turbidimetric assays of microtubule assembly were performed at 330 nm with 1, 2, or 10 mm path length cuvettes in a thermostatically controlled cell compartment of a Cary 118 or Pye-Unicam SP8000 spectrophotometer equipped with an SP8005 cell autochanger for simultaneous monitoring of up to four samples. Results are expressed as a change in apparent absorbance or optical density, ΔOD_{330}. Assembly was initiated by the addition of GTP to a concentration of ~1 mM followed by rapidly raising the temperature from 10 to 37 °C.

Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) with the exception that the pH of the electrode buffer was adjusted from 8.3 to 9.1 (Witman et al., 1976) to improve resolution of α- and β-tubulin subunits. Separating gel slabs (7.5%) overlayed with stacking gels (3.5%) were used routinely. The gels were run at a constant 35-mA current in a Uniscil/Gradipore UE2 apparatus.

For quantitative analysis the gels were stained with Coomassie Brilliant blue F and scanned with a Joyce-Loebl Model E12 MkIII microdensitometer after destaining, and bands were estimated by assuming an identical staining efficiency for all bands.

Miscellaneous. Protein concentration was determined by the method of Lowry using bovine serum albumin as the standard. The concentration of Mg^{2+} in buffers and protein solutions was measured by the method described by Williams & Detrich (1979). Samples were prepared for electron microscopy by negative staining with 1% uranyl acetate on carbon-coated grids.

Results

Electrophoretic Analysis of Tubulin Preparations. MT protein prepared by three cycles of the assembly–disassembly method was found to contain 80–85% tubulin $\alpha\beta$ heterodimer and 15–20% MAPs as judged by densitometer measurements of Coomassie Blue stained polyacrylamide gels (Figure 1a). The WT dimer (Figure 1b) was effectively free of MAPs. Phosphocellulose chromatographed MT protein preparations (PCT dimer and MgPCT dimer) are shown in parts c and d of Figure 1, respectively.

Estimations of the efficiency of the removal of the MAP fraction from phosphocellulose chromatographed MT-protein complex were made by running heavily overloaded gels. Tu-

Table 1: Far- and Near-UV CD Data

	Far-UV					
	exptl data				lit. values	
sample	λ (nm)	Δε (M^{-1} cm^{-1})	[Θ] × 10^{-3} (deg·cm^2/dmol)	SD	[Θ] × 10^{-3} (deg·cm^2/dmol)	SD
MT-protein complex	220	−2.76	−9.11	±10%		
PCT dimer	220	−2.70	−8.91	±10%		
WT dimer	220	−2.37	−7.33	±6%	−9.0[a]	±10%
					−12.0[b]	±10%
					−10.7[c]	±5%

	Near-UV					
	exptl data				lit. values	
sample	λ (nm)	Δε × 10^{-3} (M^{-1} cm^{-1})	[Θ] (deg·cm^2/dmol)	SD	[Θ] (deg·cm^2/dmol)	SD
MT-protein complex	297	1.4	4.5			
	280	−13.1	−43.2	±10%		
	260	−4.2	−13.7			
WT dimer	285	−8.7	−28.7	±15%	−35.0[d]	±25%
					−40.0[b]	±20%
					−33.0[c]	±5%
					−26.0[a]	±40%
	263	11.36	37.5	±20%	35.0[d]	±12%
					35.0[b]	±12%
					37.0[c]	±18%
					58.0[a]	
PCT dimer	280	−5.4	−17.8	±34%		
	260	1.3	4.3			
MgPCT dimer	280	−10.1	−33.3	±5%		
	260	−1.1	−3.6			

[a] Lee & Lee (1979). [b] Lee et al. (1978). [c] Appu Rao et al. (1978). [d] Lee & Timasheff (1977).

bulin eluted from phosphocellulose was found to be completely free of MAPs, with none of the characteristic HMW or τ bands detectable even at loadings in excess of 200 μg of protein.

Magnesium-presaturated phosphocellulose was initially found to be not quite as effective as normal phosphocellulose at removing MAPs. On similarly overloaded gels, HMW2, the MAP present in the greatest concentration and thus the easiest to detect, was found to constitute a maximum of 0.3% of the total protein in the worst case investigated. Even this residual amount represents at least a 50-fold reduction in HMW2 content compared to that found in MT-protein complex. The efficiency of tubulin dimer separation from MAPs on MgPC columns was improved by using double the bed volume used in normal phosphocellulose columns. This improved the efficiency of separation to that obtained with normal phosphocellulose. A comparison of parts c and d of Figure 1 reveals that the protein compositions of PCT dimer and MgPCT dimer are virtually identical with the product of the Weisenberg preparation (WT dimer) (Figure 1b), and all three are markedly purified in tubulin dimer relative to microtubule protein complex (Figure 1a). Densitometer scans of the two fractions obtained from gel filtration on Sepharose 6B show that MAPs are concentrated in the ring fraction (Figure 1e) and are effectively absent from the dimer fraction (Figure 1f).

CD Studies. (*1*) *Far-UV CD.* The far-UV spectra of MT-protein complex, PCT dimer, and WT dimer are compared in Figure 2a. In this region the CD intensity of MT-protein complex and PCT dimer is very similar (Table I). These intensities are mean values for protein concentrations of 1–2 mg/mL obtained by averaging a minimum of 12 spectra from at least five independent preparations and were reproducible to within ±10%. The WT dimer far-UV spectrum is somewhat less intense and is reproducible to within ±6% over a similar concentration range. The intensity we have obtained at 220 nm is in the lower range of values quoted in the literature. (Table I).

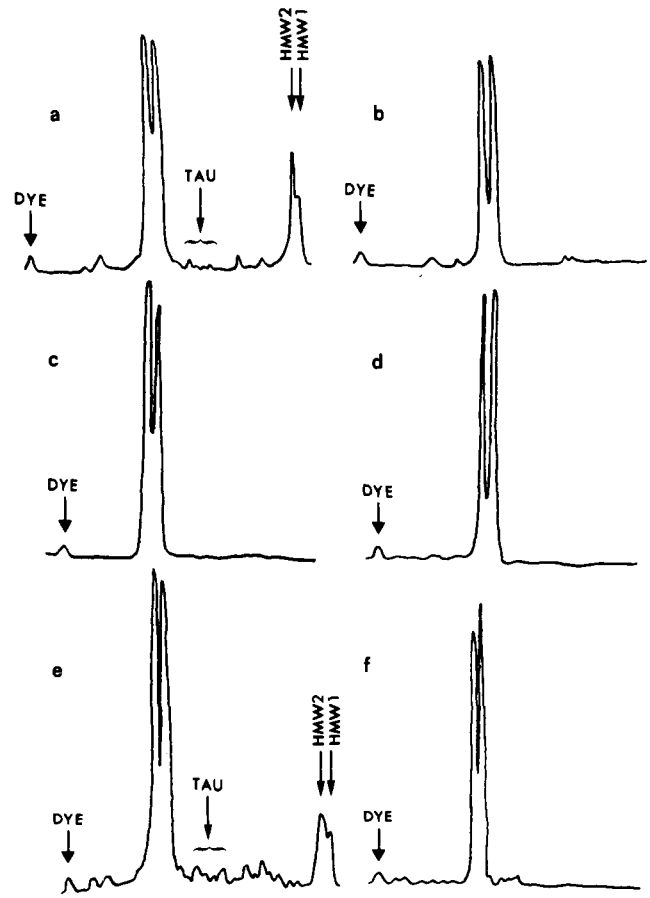

FIGURE 1: $NaDodSO_4$–polyacrylamide patterns of various tubulin preparations. (a) MT-protein complex; (b) WT dimer; (c) PCT dimer; (d) MgPCT dimer; (e) Sepharose 6B ring fraction; (f) Sepharose 6B dimer fraction.

(*2*) *Near-UV CD.* The near-UV CD spectra of MT-protein complex, PCT dimer, MgPCT dimer, and WT dimer are

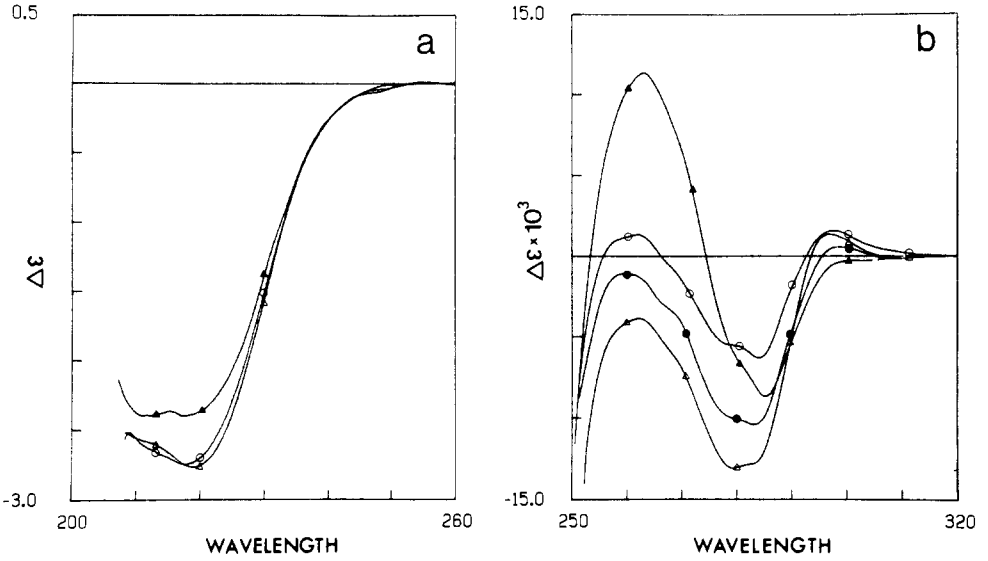

FIGURE 2: CD spectra of various tubulin preparations. (a) Far-UV CD spectra; (b) near-UV CD spectra. (△) MT protein complex; (▲) WT dimer; (○) PCT dimer; (●) MgPCT dimer. (See also Table I.)

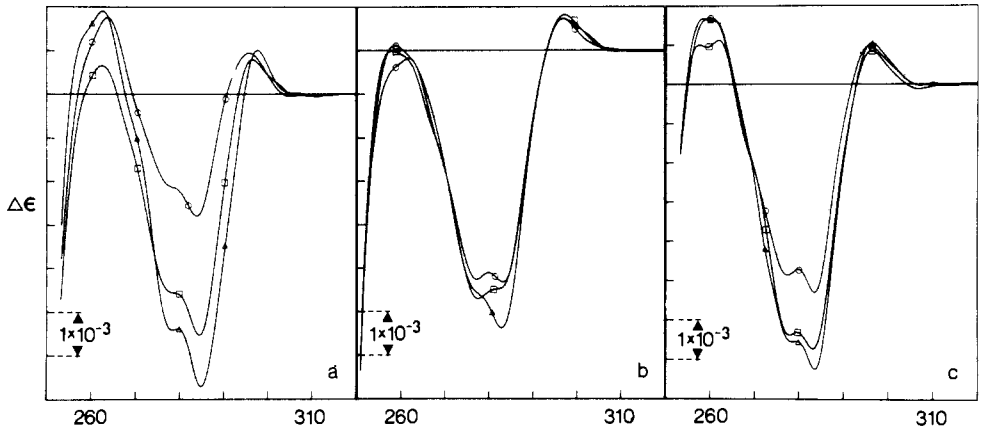

FIGURE 3: Near-UV CD spectra of PCT dimer at 37 °C and at t = 0 (△), 90 min (□), and 180 min (○). (a) PCT dimer in MEM25 buffer (see text); (b) PCT dimer in MEM25 buffer including 2.6 M glycerol. (c) PCT dimer in MEM25 buffer including 0.5 M sucrose.

compared in Figure 2b. The near-UV CD spectrum of MT-protein complex was found to be highly reproducible for a large number of independent preparations. The spectrum shows an initial small positive extremum at 297 nm, a strong negative band at 280 nm, and a turning point at 260 nm where the CD is still significantly negative (Table I).

The near-UV CD spectrum of WT dimer is well documented (Lee & Timasheff, 1977; Lee et al., 1978; Appu Rao et al., 1978). We have computed an average near-UV CD spectrum for WT dimer from 10 experimental spectra obtained from two different preparations. The result is shown in Figure 2b and is in good agreement with the published spectra of this preparation. It is clear that this spectrum differs substantially from that of MT-protein complex. Initially, we postulated that these differences were attributable to the presence of MAPs in MT-protein complex. We therefore prepared MAP-free PCT dimer by ion-exchange chromatography on phosphocellulose and confirmed the absence of MAPs (Figure 1c). However, there was still a significant difference between the near-UV CD spectrum of PCT dimer and that of WT dimer (Figure 2b). The general features of the PCT dimer spectrum resembled those of MT-protein complex more closely than those of the WT dimer, except that the intensity at 280 nm was ~50% smaller and the turning point at 260 nm was slightly positive.

We found that the intensity of the spectrum of PCT dimer prepared in this way exhibited a high degree of variability from preparation to preparation and was reproducible only to within ±34% in the 280-nm region (Table I). Several authors (Cleveland et al., 1977; Himes et al., 1977) have commented on the rather poor physical stability of PCT dimer compared with MT-protein complex. This has generally been observed as a rapid loss of its ability to reassemble after addition of a specific MAP or whole MAP fraction and loss of colchicine binding activity. This prompted us to investigate the thermal stability of PCT dimer by using near-UV CD to detect subtle changes in conformation.

The progressive change in the near-UV CD of PCT dimer at 37 °C as a function of time is shown in Figure 3a. The principle effect is loss in intensity at 280 nm. Both sucrose and glycerol are known to have a stabilizing effect on tubulin structure, and use is made of this in the preparation and storage of both MT-protein complex and WT dimer. The near-UV CD spectra show that the integrity of PCT dimer is stabilized against perturbation by temperature, most successfully by 2.6 M glycerol (Figure 3b) but almost as well by 0.5 M sucrose (Figure 3c), and it is clear that the conformation of PCT dimer is stabilized by these reagents.

It should be stressed that the progressive changes in the near-UV CD spectrum of PCT dimer shown in Figure 3a are not accompanied by parallel changes in the far-UV. This indicates that they do not reflect gross conformational changes such as alterations in α-helix and/or β-structure content of the protein, which would be detectable in this region. To give some measure of the magnitude of the conformational change depicted by Figure 3a, we calculate that the change in the

spectrum observed over 180 min at 37 °C is equivalent to the immobilization of a single aromatic residue per dimer [cf. Strickland (1974)].

Recently Williams & Detrich (1979) reported that phosphocellulose can sequester Mg^{2+} under the conditions used to separate tubulin dimer from MT-protein complex. They also showed that the polymerization of the resulting PCT dimer by addition of a Mg^{2+}-free MAP fraction was inhibited by this Mg^{2+} deficiency. They demonstrated that perturbation of the buffer components could be avoided by using Mg^{2+}-saturated columns without detriment to the efficiency of MAP binding and suggested this as an improvement of the preparative scheme.

We confirmed that the MgPCT dimer preparations were indeed free from MAPs (see above) and MgPCT dimer is found to have a characteristic near-UV CD spectrum (Figure 2b). Repeated preparations of MgPCT dimer have shown <5% variability which makes it the most reproducible preparation we have investigated (Table I).

The spectrum resembles that of MT-protein complex closely but shows small significant differences. It is known that MAPs dissociate from rings in the presence of 0.8 M NaCl (Marcum & Borisy, 1978). We therefore examined the near-UV CD spectra of MT-protein complex in the presence of 0.8 M NaCl. The resulting spectrum was virtually identical with that of MgPCT dimer (Figure 2b) suggesting that the CD contribution due to interaction with MAPs is indeed small.

In other experiments it was found that neither 3.4 M glycerol nor 0.5 M sucrose perturbs the spectra of MgPCT dimer or MT-protein complex, as was found for PCT dimer (Figure 3). Also, the thermal stability of the near-UV CD spectra with time has been investigated for MT-protein complex and MgPCT dimer. Compared to PCT dimer, the CD spectra of both were stable at 280 nm but showed a minor increase in negative CD at 260 nm after 180 min at 37 °C (not shown). Thus, the CD spectra show that MgPCT dimer is a stable species with characteristic conformation.

It is therefore of interest to see whether the CD properties of MgPCT dimer can be restored to a PCT dimer preparation by addition of Mg^{2+}. PCT dimer was made 1 mM in Mg^{2+} immediately upon elution from the column. Mg^{2+} did not restore the intensity of the spectrum to values characteristic of MgPCT dimer. Mg^{2+} enhances the thermal stability at 37 °C but is less effective than sucrose or glycerol at maintaining the integrity of the protein. The reverse process was examined by adding EDTA to MgPCT dimer to a final concentration of 10 mM to sequester the Mg^{2+}. Reductions in CD intensity at the 280- and 260-nm regions were observed, though the 260-nm turning point never became positive. Thus, interconversion between MgPCT dimer and PCT dimer is not readily reversible.

In comparing these results with the properties of WT dimer, it has previously been shown that the typical near-UV CD spectrum of WT dimer (cf. Figure 2b) is unaffected by a wide range of solvent conditions, including the addition of 16 mM Mg^{2+} and 3.4 M glycerol necessary for optimal self-assembly of WT dimer (Lee & Timasheff, 1977; Lee et al., 1978). The WT-dimer spectrum differs from the MgPCT-dimer spectrum mainly in the spectroscopic region 250–300 nm, which can reflect contributions from disulfide chromophores, and indeed some differences in sulfhydryl content have been reported for WT dimer and MT-protein complex (Mellon & Rebhun, 1976). We therefore investigated the effects of sulfhydryl reagents (β-mercaptoethanol and dithiothreitol), a sulfhydryl oxidizing agent (diamide), and mild denaturing conditions on the near-UV CD spectrum of our protein preparations. The

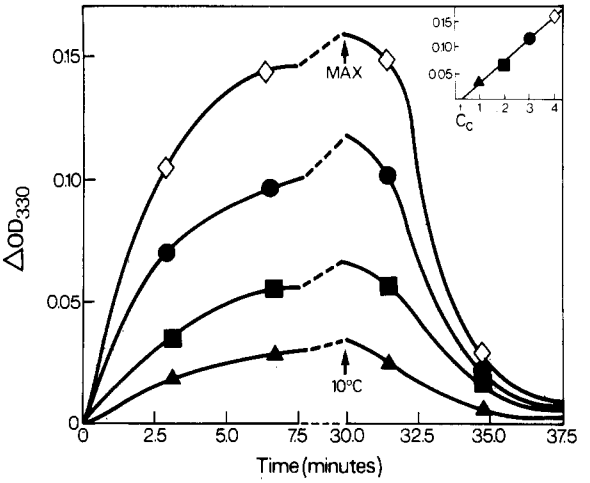

FIGURE 4: Reversible assembly–disassembly of MT-protein complex followed by turbidity (ΔOD_{330}) at protein concentrations (▲) 1.0 mg/mL; (■) 2.0 mg/mL; (●) 3 mg /mL; (◊) 4 mg/mL (for conditions see text). (Inset) ΔOD_{330}(max) vs. protein concentration showing critical protein concentration of 0.2 mg/mL.

results showed relatively minor changes with no interconversion between the spectra characteristic of the different preparations, and we conclude that the observed differences in CD are not due to different states of sulfhydryl oxidation.

Assembly Studies. The assembly properties of the various tubulin-containing fractions have been investigated in parallel with CD studies in order to look for a possible correlation between competence in reassembly and the CD spectrum reflecting the structural integrity of the protein.

The assembly characteristics of MT-protein complex isolated by our protocol have been studied in detail. Assembly was stimulated in a solution, preincubated at 37 °C by addition of GTP to a final concentration of 1 mM, and the microtubules thus formed could be depolymerized by reducing the temperature to ~15 °C. The extent of assembly at any given protein concentration above the critical concentration was found to be dependent on Mes concentration (Himes et al., 1977) and was optimal at 100 mM Mes and 1 mM GTP. Assembly was inhibited by both Ca^{2+} and EDTA, and the microtubules at 37 °C could be depolymerized by the addition of EDTA or Ca^{2+}, the latter being most effective at a concentration of 1 mM.

A typical reassembly profile is shown in Figure 4 where four different concentrations of MT-protein complex were taken through one complete cycle of assembly and disassembly. The inset shows that the critical concentration for MT-protein complex determined was ~0.15 mg/mL, consistent with values quoted in the literature.

WT dimer would not assemble in 100 mM Mes and 1 mM GTP. The minimal requirement for assembly of this protein is the use of a buffer containing 16 mM $MgCl_2$ which results in an apparent critical concentration of 8 ± 1 mg/mL, and optimum assembly is achieved in the following buffer: 10 mM Mes, 16 mM $MgCl_2$, 1 mM EGTA, 3.4 M glycerol, and 0.1 mM GTP with a critical concentration of 0.4 ± 0.1 mg/mL (Lee & Timasheff, 1977).

WT dimer was transferred to this buffer by either dialysis or G-25 Sephadex chromatography and was found to assemble satisfactorily. When the GTP concentration was increased to 0.5 mM just prior to incubation at 37 °C, the extent of assembly was increased. With the exception of a shorter lag phase for both assembly and disassembly, WT-dimer assembly profiles resemble those of MT-protein complex, although WT dimer produced a ΔOD_{330} per milligram of protein per centimeter pathlength of ~0.1 absorbance unit compared to 0.2

absorbance unit for MT-protein complex.

Attempts to assemble PCT dimer and MgPCT dimer at concentrations of up to 3 mg/mL, under the solution conditions that were optimal for MT-protein complex, were not successful. This is consistent with the quoted critical concentration for dimer chromatographed on phosphocellulose of ~6 mg/mL in the absence of MAPs and glycerol (Himes et al., 1977).

The assembly of these fractions from PC chromatography may be stimulated at concentrations below this critical concentration by addition of individual MAPs (Weingarten et al., 1975; Herzog & Weber, 1978) or by whole MAP fraction (Murphy et al., 1977). While this represents an ideal approach, it has the inherent disadvantage that negative results (i.e., no stimulation of assembly) could be a function of either assembly-incompetent dimer or nonfunctioning MAPs. Since both isolated components of this system are potentially unstable, there is no direct, independent check of the reassembly competence of the individual components.

An alternative is to use the enhancement of the assembly of ring fraction by tubulin dimer (Sloboda et al., 1976). We found that ring fraction in glycerol, isolated from a Sepharose 6B column, assembled at 37 °C in the presence of GTP but exhibited an extended lag phase prior to assembly compared with MT protein at a similar concentration. It showed reduced sensitivity to cold disassembly, with only a slight reduction in ΔOD_{330} after prolonged incubations at 10 °C, as anticipated for a preparation enriched in MAPs and assembled in the presence of glycerol. We used the assembly of this ring fraction to compare the assembly competence of the different tubulin dimer preparations.

The assay used measures the enhancement of the assembly of a fixed concentration of rings by the addition of increasing amounts of the purified tubulin dimer under investigation. The results obtained with MgPCT dimer are shown in Figure 5a, and it is seen that a progressive increase in the extent of assembly is observed, directly proportional to the amount of MgPCT dimer added. The assembled material, when examined by electron microscopy, shows an almost complete absence of rings and the presence of well-defined microtubules; amorphous material is only minimally observed. The assembled system is at least partially reversed by cold temperature [cf. Murphy et al. (1977)] and thus functions in a manner similar to that of MT-protein complex in glycerol.

The effect of PCT dimer on ring assembly is shown in Figure 5b. On a weight basis, PCT dimer enhanced ring assembly only to 18–34% of that achieved by MgPCT dimer. This evidence supports the argument that PCT dimer is Mg^{2+} deficient but also that the differences between MgPCT dimer and PCT dimer are not completely reversed by addition of Mg^{2+}.

In similar experiments, the ability of WT dimer to enhance the level of assembly of rings was examined. No significant incorporation of WT dimer into microtubules apparently occurred as far as was detectable by changes in ΔOD_{330}. However, if these samples were made 16 mM in Mg^{2+} after the rings had attained their plateau assembly value, a separate phase of assembly of WT dimer was observed. Thus, WT dimer showing normal capacity for self-assembly in 16 mM Mg^{2+} was significantly less effective than MgPCT dimer in assembly seeded by ring fraction.

Discussion

We have compared the near-UV CD and assembly properties of MT-protein complex prepared by the assembly–disassembly of microtubules and tubulin dimer prepared from this MT-protein complex by phosphocellulose ion-exchange chromatography (MgPCT dimer and PCT dimer). We have also verified certain properties of the WT dimer prepared by the fractionation procedure of Weisenberg & Timasheff (1970).

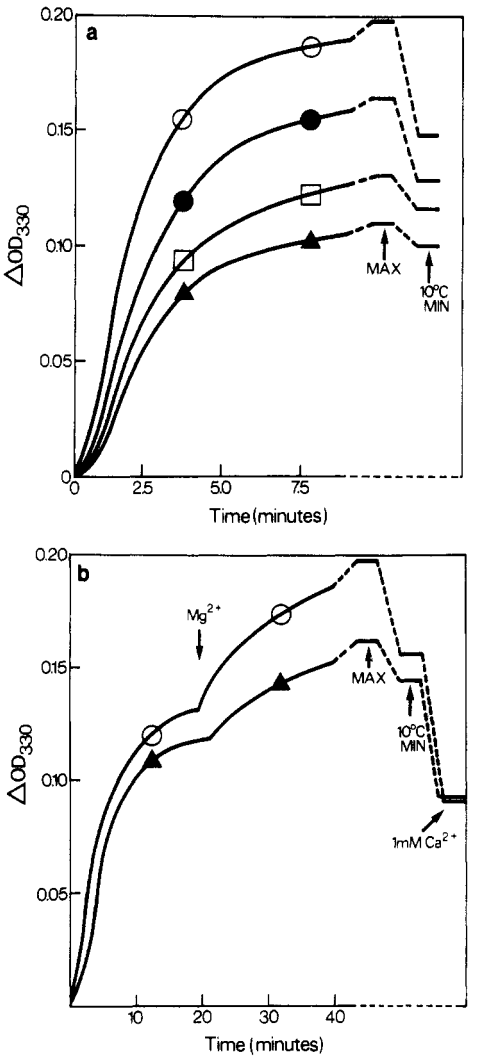

FIGURE 5: Enhancement of assembly of ring fraction by MgPCT dimer and PCT dimer. (a) Ring fraction (0.61 mg/mL) incubated at 37 °C with additions of (▲) 0, (□) 0.1 mg/mL, (●) 0.25 mg/mL, and (○) 0.41 mg/mL MgPCT dimer. Maximum turbidity is as indicated; also shown is the effect of reducing the temperature to 10 °C. (b) Ring fraction (0.68 mg/mL) incubated at 37 °C with addition of (▲) 0 and (○) 0.33 mg/mL PCT dimer. Addition of Mg^{2+} (1 mM) and maximum turbidity are as indicated; also shown is the effect of reducing the temperature to 10 °C and the subsequent addition of 1 mM Ca^{2+}.

The electrophoretic profiles of all the dimer preparations were essentially indistinguishable and showed the effective removal of MAPs from MT-protein complex by phosphocellulose chromatography. Thus MgPCT dimer and PCT dimer are essentially MAP-free tubulin, as is the WT-dimer preparation. Likewise, the dimer fraction from Sepharose 6B is also free of MAPs, which, as expected, are considerably concentrated in the ring fraction. This ring fraction thus provides a useful source of MAPs which shows no instability at 25 °C as judged by the constancy of the CD spectrum, the absorption spectrum, and the ability to seed other assembly systems.

Removal of MAPs from MT-protein complex by PC chromatography produces tubulin dimer whose properties are dependent upon the concentration of Mg^{2+} present during the separation procedure. Thus PCT dimer is rather unstable, and the CD spectra show loss of native structure in the near-UV

which occurs before extensive changes in secondary structure. MgPCT dimer, by contrast, is more stable. These results correlate well with the observed loss of assembly capability of Mg-depleted PCT dimer (Cleveland et al., 1977; Himes et al., 1977) and provide an initial suggestion that assembly capacity is strongly affected by changes in conformation, which we discuss further below. The variability and instability detected in the near-UV CD studies with PCT dimer led us to adopt a new protocol for the removal of MAPs from MT-protein complex, using the Mg-pretreated phosphocellulose procedure of Williams & Detrich (1979). The MgPCT dimer produced by this method is the main object of discussion here, and we believe that the MgPCT dimer is the most characteristic native form of the tubulin dimer as found in the MT-protein complex, based on the following arguments.

(*1*) *MgPCT Dimer Closely Resembles MT-Protein Complex in Its Near-UV CD.* This spectroscopic parameter is an extremely sensitive indicator of conformational fine structure. The small but reproducible difference appears to be significant. It might result from a small modulation of tubulin dimer conformation due to interaction with MAPs, or it might reflect a minor contribution from MAP near-UV CD, though this has not yet been characterized independently. It is interesting that the near UV CD of Sepharose 6B tubulin dimer superimposes almost exactly upon that of MgPCT dimer (Clark et al., 1980).

(*2*) *MgPCT Dimer Shows a Thermal Stability Close to That of MT-Protein Complex.* Removal of Mg^{2+} is known to induce instability, probably due to irreversible processes. The conformational instability of PCT dimer with temperature appears to be arrested but not reversed by addition of Mg^{2+}.

(*3*) *MgPCT Dimer Assembles Efficiently with Rings.* The assembly of MgPCT dimer in the presence of rings resembles that of MT-protein complex. The assembled material shows predominantly intact microtubules, and the process is partially reversed by cold temperature. By contrast, PCT dimer assembled much less efficiently than MgPCT dimer in this system. The 25% increment of turbidity (per milligram of protein) could be improved to 60% by inclusion of 1 mM Mg^{2+}, indicating a partial irreversible loss of assembly capability during PC chromatography.

(*4*) *MgPCT Dimer Differs Substantially from WT Dimer in Near-UV CD Properties.* Recognition of the importance of the Mg^{2+} pretreatment procedure has led to a reliable and reproducible method for obtaining MAP-free tubulin dimer. This MgPCT dimer resembles MT-protein complex but differs substantially from the well-characterized WT dimer obtained by fractionation procedures. The electrophoresis patterns of Figure 1 exclude the possibility of limited proteolytic cleavage, and there is no evidence of primary structural modification of these preparations. Simple chemical treatment and a range of solvent conditions do not lead to interconversion of the CD spectra, and we conclude that these spectra are characteristic of distinctive species, representing different conformational modifications of the tubulin dimer. The magnitude of the difference in CD suggests significant spectroscopic changes in a few aromatic residues only, and we infer that these represent a localized conformational change.

Role of Tubulin Conformation in Microtubule Assembly. In comparing the assembly capabilities of different tubulin preparations, both in this work and in the literature, it is necessary to specify carefully the conditions under which assembly has been assayed. WT dimer optimally forms microtubules in the presence of 3.4 M glycerol and 16 mM Mg^{2+}, conditions which evidently promote nucleation and facilitate growth of microtubule-like structures. These physical conditions do not change the conformation of disassembled WT dimer, and the WT dimer can be recovered in its original conformation after cold disassembly of WT microtubules. By contrast, the MgPCT dimer, which is similar in conformation to MT-protein complex, differs substantially from WT dimer. It too may be recovered in its original conformation after cold disassembly of microtubules assembled from MT protein. We conclude that the assembled WT dimer and the assembled MT-protein complex differ to the extent that they contain dimer possessing the different conformations of WT dimer and MgPCT dimer, respectively.

The main difference in assembly properties is the low critical concentration for assembly of MT-protein complex in low Mg, low glycerol buffers compared with conditions for WT-dimer self-assembly. The difference is attributable to the presence of complexes of tubulin dimer with MAPs which can act as efficient nucleation sites for assembly. Rings appear to act as an effective source of such nucleation complexes for seeding the assembly. Thus a critical comparison of tubulin dimer preparations can be made by using the assay system based upon assembly of ring fraction in which nucleation is demonstrably efficient. It is clear that MgPCT dimer functions normally in this system and that the mixture (rings plus MgPCT dimer) effectively reproduces the MT-protein complex assembly system. The fact that WT dimer does not enhance the standard ring assembly assay indicates different assembly properties, even in the presence of nucleating material. The addition of 16 mM Mg^{2+} restores conditions under which WT dimer undergoes self-assembly. Thus the conformational difference in WT dimer appears to affect the capability for microtubule growth in a seeded assembly system and therefore implies a reduced interaction of WT dimer with some component (possibly Mg^{2+} itself) under the low Mg^{2+} conditions of the assay. The requirement for high Mg^{2+} for assembly of WT dimer appears necessary to compensate for the changed conformation of WT dimer relative to MgPCT dimer. However, under these conditions the typical conformation of WT dimer is maintained. These arguments have important implications for copolymerization studies which are presently being developed.

The characterization of MgPCT dimer reported here has implications for the interpretation of numerous in vitro assembly studies reported in the literature. Thus, the seeded assembly of 6S tubulin, prepared as a high-speed supernatant from a (glycerol-free) preparation of microtubule-protein complex, proceeds with high efficiency [see Scheele & Borisy (1979)], and we infer that this tubulin dimer has a conformation comparable to MgPCT dimer. The present work provides the additional criterion of near-UV CD which may be used as a correlate of the ability of tubulin dimer preparations to promote microtubule elongation in an in vitro assembly system.

References

Amos, L. A. (1977) *J. Cell Biol. 72*, 642–654.

Appu Rao, A. G., Hare, D. L., & Cann, J. R. (1978) *Biochemistry 17*, 4735–4739.

Clark, D. C., Martin, S. R., & Bayley, P. M. (1980) *Biochem. Biophys. Res. Commun. 97*, 628–634.

Cleveland, D. W., Hwo, S.-Y., & Kirschner, M. W. (1977) *J. Mol. Biol. 116*, 207–225.

Diehl, H., & Lindstrom, F. (1959) *Anal. Chem. 31*, 414–418.

Dustin, P. (1978) in *Microtubules*, Springer, Berlin, Heidelberg, and New York.

Erickson, H. P. (1974) *J. Supramol. Struct. 2*, 393–411.