Jovin, T., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9,* 351.
Kamen, M. D., Bartsch, R. G., Horio, T., and de Klerk, H. (1963), *Methods Enzymol. 6,* 391.
Kamen, M. D., Dus, K. M., Flatmark, T., and de Klerk, H. (1971), in Electron and Coupled Energy Transfer in Biological Systems, King, T. E., and Klingenberg, M., Ed., New York, N.Y., Marcel Dekker, p 243.
Keller, R. M., Pettigrew, G. W., and Wuthrich, K. (1973), *FEBS Lett. 36,* 151.
Laemmli, U. K. (1970), *Nature (London) 227,* 680.
Lemberg, R., and Barrett, J. (1973), Cytochromes, London and New York, Academic Press.
McDonald, C. C., and Phillips, W. D. (1973), *Biochemistry 12,* 3170.
McDonald, C. C., Phillips, W. D., and LeGall, J. (1974), *Biochemistry 13,* 1952.
Margoliash, E., and Schejter, A. (1966), *Adv. Protein Chem. 21,* 113.
Nagel, G. W., and Schachman, H. K. (1975), *Biochemistry 14,* 3195.
Redfield, A. G., and Gupta, R. K. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 405.
Salemme, F. R., Freer, S. T., Xuong, N. H., Alden, R. A., and Kraut, J. (1973), *J. Biol. Chem. 248,* 3910.
Sano, S., Nanzyo, N., and Rimington, C. (1964), *Biochem. J. 93,* 270.
Sherman, F., Stewart, J. W., Parker, J. H., Inhaber, E., Shipman, N. A., Putterman, J., Gardisky, R. L., and Margoliash, E. (1968), *J. Biol. Chem. 243,* 5446.
Slama, J. T., Smith, H. W., Willson, C. G., and Rapoport, H. (1975), *J. Am. Chem. Soc. 97,* 6556.
Smith, G. M., and Kamen, M. D. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4303.
Smith, W. R., Sybesma, C., Litchfield, W. J., and Dus, K. (1973), *Biochemistry 12,* 2665.
Sutherland, J. C., Vickery, L. E., and Klein, M. P. (1974), *Rev. Sci. Instrum. 45,* 1089.

In Vitro Reconstitution of Calf Brain Microtubules: Effects of Solution Variables†

James C. Lee‡ and Serge N. Timasheff*

ABSTRACT: The effects of solution variables on the in vitro reconstitution of calf brain tubulin, purified by the method of Weisenberg et al. (Weisenberg, R. C., Borisy, G. G., and Taylor, E. W. (1968), *Biochemistry 7,* 4466–4479; Weisenberg, R. C., and Timasheff, S. N. (1970), *Biochemistry 9,* 4110–4116), as modified by Lee et al. (Lee, J. C., Frigon, R. P., and Timasheff, S. N. (1973), *J. Biol. Chem. 248,* 7253–7262), were investigated at pH 7.0. Reconstitution of microtubules was successful in a variety of buffer systems, the free energy of the propagation step of microtubule formation being little dependent on the buffer. Microtubule formation is promoted by magnesium ions and guanosine triphosphate, but inhibited by calcium ions. The dependence of the apparent association constant for microtubule formation on ligand concentration was analyzed by the linked function theory of Wyman (Wyman, J. (1964), *Adv. Protein Chem. 19,* 224–286), leading to the conclusion that the formation of a tubulin–tubulin contact involves the binding of one additional magnesium ion per tubulin dimer. Microtubule formation is also accompanied by the apparent binding of one additional proton and the release of water molecules, as suggested by the thermodynamic parameters determined. The reaction is entropy driven with an apparent heat capacity change, ΔC_p, of -1500 ± 500 cal/deg-mol. The enhancement of tubulin reassembly by glycerol is most likely due to nonspecific protein–solvent general thermodynamic interactions.

Following the initial observations of Weisenberg (1972) that microtubules can be reconstituted from a partially purified tubulin preparation, a number of reports have appeared on the in vitro reconstitution of microtubules (Olmsted and Borisy, 1975; Jacobs et al., 1974, 1975; Shelanski et al., 1973; Kirschner et al., 1974; Erickson, 1974). In all of these studies, the protein was prepared according to several variants of the polymerization–depolymerization (cycle) procedure of Shelanski et al. (1973). This procedure yields tubulin of 80 to 90% purity, the rest consisting of 200 000 to 300 000 molecular-weight components and/or smaller components distributed into 10 to 15 different protein bands observed in sodium dodecyl sulfate gel electrophoresis (Weingarten et al., 1975; Erickson, 1974; Murphy and Borisy, 1975). Results obtained with tubulin prepared by the cycle procedure have led Kirschner and co-workers (Kirschner et al., 1974; Kirschner and Williams, 1974) to conclude that the 5.8S native dimers of tubulin (110 000 molecular weight) are chemically and functionally different from the subunits which make up ring structures, that the ring structures are not in equilibrium with the 5.8S species, and that only rings polymerize into microtubules, the 5.8S species being unable to do so. Furthermore, these authors have asserted that the ability of the rings to assemble into microtubules is due to a salt-dissociable factor. In the absence of such a factor, they have stated that "tubulin is utterly unable to polymerize into microtubules" (Weingarten et al., 1975). These conclusions are in contrast with the results obtained with

† From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received July 28, 1976.* Publication No. 1137. Supported by National Institutes of Health Grants GM-14603 and CA-16707, National Science Foundation Grant BMS 72-02572, and a grant from the American Cancer Society, Massachusetts Division.

‡ Present address: Department of Biochemistry, St. Louis University, St. Louis, Missouri 63104.

tubulin purified by the Weisenberg procedure (Weisenberg et al., 1968, 1970). Using such a tubulin preparation, which is approximately 99% pure by the criterion of sodium dodecyl sulfate gel electrophoresis, it has been shown that the 5.8S dimers are capable of existing in dynamic equilibrium both with double rings (Frigon et al., 1974; Frigon and Timasheff, 1975a,b), which appear to be of identical structure with those reported as products of microtubule depolymerization (Erickson, 1974; Kirschner and Williams, 1974), and with microtubules (Lee et al., 1975; Lee and Timasheff, 1975; Timasheff et al., 1976a). In such preparations, all of the 5.8S tubulin dimeric molecules behave in a thermodynamically identical manner with respect both to the magnesium-induced self-association to rings (Frigon and Timasheff, 1975a,b), and to the self-assembly to reconstituted microtubules (Lee and Timasheff, 1975). The in vitro reconstitution of microtubules from tubulin prepared by the Weisenberg procedure has been shown to procede in the absence of any high-molecular-weight species (Lee and Timasheff, 1975), be they the 200 000–300 000 molecular-weight proteins found in some cycle preparations (Murphy and Borisy, 1975), but not in others (Weingarten et al., 1974), or preexisting nucleation centers which have been claimed as essential to reconstitution (Borisy and Olmsted, 1972), and have been identified as single rings with an outside diameter of 29 nm (Borisy and Olmsted, 1972) or 35 nm (Olmsted et al., 1974).

The composition of the reconstitution medium has also been reported to affect strongly the process of tubulin self-assembly to microtubules. Working with cycle preparations, Olmsted and Borisy (1975) have reported that microtubule polymerization is promoted by buffers which possess strong dipole moments but that "no assembly occured in other commonly used buffers (e.g., phosphate)". On the basis of this, they have proposed that zwitterions might be involved in microtubule regulation in vivo. On the other hand, tubulin highly purified by the Weisenberg procedure has been reconstituted into microtubules in phosphate buffer (Lee and Timasheff, 1975). While GTP[1] is normally used in microtubule reconstitution media, Shelanski et al. (1973) have reported that, in the presence of glycerol, no additional GTP is required for microtubule formation.

In view of these apparently contradictory statements in the literature, it seemed desirable to investigate systematically the effects of solution variables on the in vitro reconstitution of microtubules from tubulin preparations essentially devoid of other proteins. The results of studies on the effects of temperature, buffer composition, ligands, and glycerol are reported in this paper.

Materials and Methods

Glycine, cacodylate, β-alanine, lysine, Tris, Mes, and Pipes were purchased from Sigma Chemical Co. Imidazole and reagent-grade glycerol were obtained from Eastman Kodak Co. and Fisher Scientific Co., respectively. Uranyl acetate was obtained from Mallinckrodt Chemical Works and [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) was from J. T. Baker Chemical Co.

Calf brain tubulin was prepared by the modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg and Timasheff, 1970; Lee et al., 1973). The concentration of protein was determined by diluting the sample with 6 M guanidine hydrochloride and measuring the absorbance at 274 nm using an absorptivity of 1.15 mL/mg-cm (Lee et al., 1973).

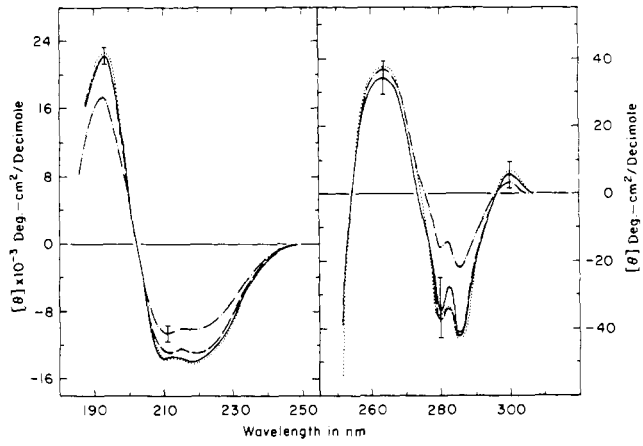

FIGURE 1: Circular dichroism spectra of tubulin. The corresponding symbols and conditions are (—) tubulin in the following, pH 7.0, 10^{-2} M buffer systems: phosphate, Mes, Pipes, Tris, imidazole, cacodylate, and glycine with 10^{-4} M GTP, 21–23 °C, and in 3.4 M glycerol, 10^{-2} M phosphate, 1.6×10^{-2} M $MgCl_2$, 10^{-4} M GTP, 10^{-3} M EGTA at pH 7.0, 37 °C; (- • -) in 10^{-2} M glycine at pH 7.0, in the absence of GTP, 21–23 °C; (· · ·) in 3.4 M glycerol, 10^{-2} M phosphate, 1.6×10^{-2} M $MgCl_2$, 10^{-4} M GTP, 10^{-3} M EGTA at pH 7.0, 21–23 °C; (- - -) in 0.2 M Pipes at pH 7.0, 21–23 °C. Protein concentrations were 1.2 to 1.6 mg/mL. The bars represent experimental uncertainties.

The self-assembly of tubulin was monitored by turbidity measurements (Gaskin et al., 1974; Berne, 1974) and by electron microscopy. The assembly buffer, unless specified otherwise, consisted of 1.6×10^{-2} M $MgCl_2$, 3.4 M glycerol, 10^{-3} M EGTA, and 10^{-4} M GTP and the particular buffering ion at pH 7.0. Turbidity was monitored at 350 nm on Cary 14 or 118 recording spectrophotometers. The protein solutions were incubated at 37 °C or other specified temperatures in a water-jacketed cuvette, which was thermostatically regulated by a Haake KT 33 or Neslab RTE-4 circulator. The activities of ionic species were calculated as the mean ion activities, $a_{\pm}$, properly taking into account the effect of glycerol on the activity coefficients.

Results

Buffer Composition. Since solvent components may affect the tubulin self-assembly reaction either by inducing conformational changes or by thermodynamic interactions, the effects of changes in buffer composition were monitored both by circular dichroism and by measurements of the apparent equilibrium constant for the addition of tubulin subunits to growing microtubules, defined as the reciprocal of the observed critical concentration. The results of the circular dichroism experiments are shown in Figure 1. It was found that the CD spectra obtained in pH 7.0, 0.01 M phosphate, Mes, Pipes, Tris, imidazole and cacodylate buffers were identical within experimental error whether GTP was present or not. The average of the CD spectra obtained in these buffers is shown by the solid line of Figure 1. The CD spectrum in 0.2 M Pipes, shown by the dashed line, can also be regarded as identical within experimental error with those obtained at lower buffer concentrations. The only buffer system which gave a spectrum differing from the others was 0.01 M, pH 7.0, glycine in the absence of GTP, in which the intensity, but not the position of several bands, changed. The cause of this spectral difference is not known. It might be pertinent to remark that similar

[1] Abbreviations used are: Mes, 4-morpholineethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; GTP, guanosine triphosphate; CD, circular dichroism.

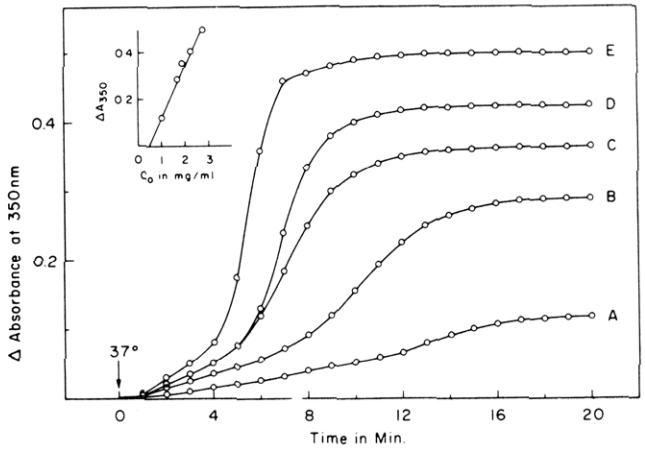

FIGURE 2: Effect of tubulin concentration on development of turbidity on heating to 37 °C. The solvent was 3.4 M glycerol, 10^{-2} M glycine, 10^{-4} M GTP, 10^{-3} M EGTA, 1.6×10^{-2} M $MgCl_2$, pH 7.0. The protein concentrations were: (A) 1.05 mg/mL; (B) 1.70 mg/mL; (C) 1.89 mg/mL; (D) 2.23 mg/mL; and (E) 2.78 mg/mL.

changes have been observed in the case of ribonuclease in the presence of carboxylic acids (Cann, 1971). Addition of 10^{-4} M GTP to the glycine buffer resulted in a CD spectrum identical with those shown by the solid line of Figure 1. It would appear, therefore, that the reported difference between the ability of tubulin to reconstitute in phosphate and zwitterionic buffers does not reside in any significant conformational differences of the protein in these systems.

The effects of buffer composition on the thermodynamics of microtubule reconstitution were examined by the turbidimetric method of Gaskin et al. (1974). This has been shown (Berne, 1974) to be, within a defined set of assumptions, a rigorously valid quantitative method for measuring the extent of polymerization when the product has the geometry of long thin rods. Extrapolation of turbidity values obtained at different protein concentrations to zero turbidity gives the critical concentration, C_r. Oosawa and Higashi (1967) have shown that, for helical polymerization, such as that of actin, or, indeed, of the tubulin self-assembly to microtubules (Gaskin et al. (1974), the critical concentration is equal within close approximation to the inverse of the association constant, K_p, for the addition of each subunit to a growing helix.[2]

Figure 2 shows typical results of changes in turbidity when a tubulin solution in 0.01 M glycine reconstitution buffer is heated to 37 °C. The turbidity values in the plateau regions are proportional to the tubulin concentrations, as shown in the inset of Figure 2. Extrapolation to zero turbidity gives a value of the critical concentration, C_r, of 0.5 mg/mL, or 4.55×10^{-6} M tubulin. This results in $K_p \approx 2.2 \times 10^5$ L/mol, and an apparent free energy of polymer propagation, $\Delta G°_p = -7.6$ kcal/mol. Electron microscopic examination of the products of polymerization in this association buffer reveals the presence of microtubules, as shown in Figure 3A, although ring-like structures are also seen to be present. Apparently, the number of rings observed per grid area is greater for the glycine association buffer than other systems, such as phosphate or imidazole, shown in Figure 3B.

The results of similar turbidimetric measurements in various buffer systems are summarized in Table I. They indicate that

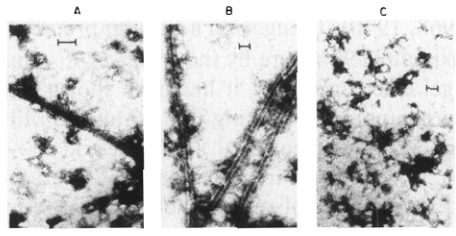

FIGURE 3: Electron micrographs of tubulin. (A) Reconstituted microtubules in 3.4 M glycerol, 10^{-2} M glycine, 1.6×10^{-2} M $MgCl_2$, 10^{-4} M GTP, 10^{-3} M EGTA, pH 7.0, at 37 °C. The magnification factor is 46 250×. (B) Tubulin in the imidazole assembly buffer, pH 7.0, at 37 °C. The magnification factor is 27 500×. (C) Tubulin in phosphate assembly buffer without GTP, pH 7.0, at 37 °C. The magnification factor is 29 250×. The bars represent 50 nm.

TABLE I: Dependence of K_p on Buffer Systems[a] at 10^{-2}M, 37 °C, and pH 7.0.

Buffer	C_r (mg/mL)	K_p (L/mol)	$-\Delta G°_{app}$ (kcal/mol)
Phosphate	1.2 ± 0.1	$9 \pm 1 \times 10^4$	7.0 ± 0.1
Tris	1.2 ± 0.1	$9 \pm 1 \times 10^4$	7.0 ± 0.1
Cacodylate	2.0 ± 0.1	$5.5 \pm 0.3 \times 10^4$	6.7 ± 0.1
Mes	0.4 ± 0.1	$28 \pm 8 \times 10^4$	7.7 ± 0.2
Pipes	0.6 ± 0.1	$18 \pm 4 \times 10^4$	7.4 ± 0.2
Imidazole	0.7 ± 0.1	$16 \pm 2 \times 10^4$	7.4 ± 0.1
Glycine	0.5 ± 0.1	$22 \pm 6 \times 10^4$	7.6 ± 0.2
β-Alanine	0.6 ± 0.1	$18 \pm 4 \times 10^4$	7.4 ± 0.2
Lysine	0.7 ± 0.1	$16 \pm 2 \times 10^4$	7.4 ± 0.1

[a] Contain 3.4 M glycerol, 10^{-4}M GTP, 10^{-3}M EGTA, and 1.6×10^{-2}M $MgCl_2$.

the polymerization of tubulin into microtubules is little dependent on the nature of the buffer components, since the spread in the polymer propagation free energies in the various solvents is not greater than 10%. Closer scrutiny does reveal some differences. It appears that chain propagation is somewhat weaker in phosphate, Tris, and cacodylate buffers of identical concentration than in the others. There is, however, no obvious systematic difference between the nature of ions in the two classes. The weaker association buffer family consists of one cationic and two anionic buffers. The buffers in which association is stronger contain all the zwitterionic species and imidazole, which is a cationic buffer. An explanation might possibly be found in the different ability of these various buffers to chelate divalent cations, although no systematic data are available on this point. It does not seem likely, however, that this small difference can reflect specific effects, such as would be expected in the case of the proposed physiological regulation (Olmsted and Borisy, 1975).

Examination of the effects of buffer concentration and ionic strength on K_p gave the results presented in Table II. It is evident that a tenfold increase in the concentration of the buffer constituent for glycine and 20-fold for Pipes does not lead to any significant change in K_p. In contrast, addition of 0.1 M NaCl to 0.01 M phosphate lowered the value of K_p threefold to 3×10^4 L/mol. These observations are in disagreement with those of Olmsted and Borisy (1975) who reported that a tubulin preparation purified by an assembly–disassembly procedure had an optimal Pipes concentration for microtubule formation of 0.1 M, the extent of polymerization decreasing in both 0.008 and 0.2 M Pipes to about 30% of that in 0.1 M Pipes. Since in protein purified by the Weisenberg procedure

[2] It is easy to show that this is valid for any mode of polymerization in which chain growth proceeds by a cooperative mechanism. In fact, the existence of a "critical concentration" is a trivial consequence of the cooperative nature of chain propagation.

TABLE II: Effects of Buffer Concentration on K_p of Calf Brain Tubulin at pH 7.0 and 37 °C.

Buffer Concn	C_r (mg/mL)	K_p (L/mol)
NaCl[a]		
0.0 M	1.2 ± 0.1	9 ± 1 × 10^4
0.1 M	3.4 ± 0.1	3.2 ± 0.1 × 10^4
Glycine[b]		
0.01 M	0.5 ± 0.1	22 ± 4 × 10^4
0.10 M	0.5 ± 0.1	22 ± 4 × 10^4
Pipes[b]		
0.01 M	0.7 ± 0.1	16 ± 2 × 10^4
0.10 M	0.6 ± 0.1	18 ± 2 × 10^4
0.20 M	0.8 ± 0.1	14 ± 2 × 10^4

[a] Buffer contains 3.4 M glycerol, 10^{-2}M phosphate, 10^{-3}M EGTA, 10^{-4}M GTP, and 1.6 × 10^{-2}M $MgCl_2$. [b] Buffers contain all the solvent constituents as in footnote a, with phosphate replaced by these buffer components.

the CD spectrum (Figure 1) and K_p (Table II) were independent of Pipes concentration, indicating the absence both of conformational and thermodynamic effects, the drastic decrease in the extent of polymerization in 0.2 M Pipes reported by Olmsted and Borisy (1975) for the assembly-disassembly prepared protein might be related to dissociation from complexation with tubulin of the heavy molecular-weight components present in such tubulin preparations. Indeed, Murphy and Borisy (1975) have reported that these components, which stimulate the formation of microtubules, are completely dissociated from tubulin in 0.3 M KCl.

Effect of Temperature. The effect of temperature on tubulin self-association was examined in the pH 7.0, 10^{-2} M phosphate buffer system. The results are shown in Figure 4 and Table III. A pronounced curvature is observed in the van't Hoff plot. The data were, therefore, fit to the equation

$$\ln K_p = a + b(1/T) + c \ln T \quad (1)$$

which is a truncated form of the integrated van't Hoff equation (Glasstone, 1947). The values of the free energy, $\Delta G°$, the enthalpy, $\Delta H°$, the entropy, $\Delta S°$, and the heat capacity, ΔC_p, changes in the reaction are given by

$$\Delta G° = -RT \ln K_p^{app}$$
$$\Delta H° = R(cT - b)$$
$$\Delta S° = (\Delta H° - \Delta G°)/T$$
$$\Delta C_p = Rc \quad (2)$$

The resulting thermodynamic parameters are summarized in Table III. The polymerization of tubulin is characterized by an apparent change in heat capacity, ΔC_p of −1500 cal/mol deg, and positive changes in enthalpy and entropy. These values of the thermodynamic parameters suggest the loss of ordered water on the formation of each tubulin–tubulin contact. They are qualitatively consistent with thermodynamic parameters observed for the in vivo formation of the mitotic spindle (Zimmerman and Marsland, 1964; Salmon, 1975a) and with the known depolymerization of microtubules both in vivo and in vitro upon application of high pressures (Marsland, 1970; Salmon, 1975b).

Effects of Ligands. With the knowledge that buffer systems per se have little effect on microtubule formation, the solvent composition requirements for the reaction were examined. It

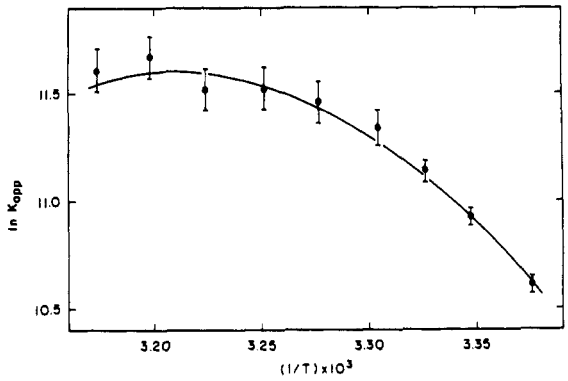

FIGURE 4: Van't Hoff plot of tubulin reconstitution. The solvent was the phosphate assembly buffer, pH 7.0.

TABLE III: Thermodynamics of Microtubule Growth in the Presence of Magnesium.[a]

T (°C)	ln K_{app}	$\Delta H°_{app}$ (kcal/mol)	$\Delta S°_{app}$ (eu)
23.0	10.62	22.79	98
25.5	10.96	19.10	86
27.5	11.18	16.16	76
29.5	11.35	13.21	66
32.0	11.47	9.52	54
34.5	11.51	5.83	42
37.0	11.51	2.15	30
39.5	11.68	−1.54	18
42.0	11.61	−5.20	6

[a] $\Delta C_{p,app}$ = 1500 ± 500 cal/mol deg.

is known that microtubule formation in vitro is strongly enhanced by the presence of magnesium ions and GTP (Lee and Timasheff, 1975), that it is inhibited by calcium ions (Weisenberg, 1972), and that it occurs to a greater extent at pH 6.5 than 7.0 (Gaskin et al., 1974). A quantitative study of the effect of these ligands on microtubule growth was undertaken. Using again the turbidity assay, the critical concentration for the reassembly reaction was determined as a function of ligand concentration, and the data were analyzed in terms of the linked function relations developed by Wyman (1964), and extended by Tanford (1969) and by Aune et al. (1971).

According to the linked function theory, it can be shown that, if the polymer growth constant observed, K_p, is purely an association constant, with no secondary reactions such as conformational changes involved, it can be expressed as a function of solvent variables at constant temperature and pressure, $K = K(a_H, a_w, a_x)$, where a_i is the activity of species i, with H representing protons, w representing water, and x representing other solute species. Then, any change in the free energy of the reaction, ΔG, can be expressed as

$$d(\Delta G) = \left(\frac{\partial \Delta G}{\partial \mu_H}\right)_{\mu_w,\mu_x} d\mu_H + \left(\frac{\partial \Delta G}{\partial \mu_w}\right)_{\mu_H,\mu_x} d\mu_w + \left(\frac{\partial \Delta G}{\partial \mu_x}\right)_{\mu_H,\mu_w} d\mu_x \quad (3)$$

where μ_i is the chemical potential of component i, $\mu_i = \mu°_i + RT \ln a_i$. If the activities of all ligands, except one, are held constant, rearrangement of eq 3 gives (Aune et al., 1971)

$$-\frac{1}{RT}\frac{d\Delta G}{d \ln a_x} = \frac{d \ln K}{d \ln a_x} = \Delta \bar{\nu}_{pref} \quad (4)$$

TABLE IV: Dependence of Microtubule Growth on Magnesium Concentration.

$[Mg^{2+}]$, Total (mol/L)	$K_{app} = 1/C_{crit}$ (L/mol)	K_2 (L/mol)
0.5×10^{-2}	4.17×10^4	1.86×10^5
0.7×10^{-2}	4.90×10^4	1.61×10^5
1.0×10^{-2}	7.03×10^4	1.74×10^5
1.2×10^{-2}	8.33×10^4	1.84×10^5
1.4×10^{-2}	9.39×10^4	1.89×10^5
1.6×10^{-2}	10.80×10^4	1.90×10^5
2.0×10^{-2}	12.93×10^4	2.13×10^5

where $\Delta\bar{\nu}_{pref}$ is the difference between the preferential interaction of solvent components with the two end states of the reaction in question. In standard multicomponent thermodynamic notation (Casassa and Eisenberg, 1964; Timasheff, 1973a), this parameter can be expressed as

$$\Delta\bar{\nu}_{pref} = \left(\frac{\partial m_x}{\partial m_p}\right)_{\mu_x}^{product} - \left(\frac{\partial m_x}{\partial m_p}\right)_{\mu_x}^{reactant} \quad (5)$$

where m_i is concentration in molal units and the subscript p refers to protein. In eq 5, the molality of protein should be expressed in terms of units of identical molecular weight in the product and the reactant. Preferential interaction, expressed in terms of preferential binding, is related to the absolute interactions of ligand and water with protein, $\bar{\nu}_i$, by (Inoue and Timasheff, 1972).

$$\left(\frac{\partial m_x}{\partial m_p}\right)_{\mu_x} = \bar{\nu}_x - \frac{m_x}{m_{H_2O}}\bar{\nu}_{H_2O} \quad (6)$$

Therefore, for the microtubule growth reaction, the dependence of the polymer propagation constant, K_p, on ligand activity is

$$\frac{d \ln K_p}{d \ln a_x} = \bar{\nu}_x{}^P - \bar{\nu}_x{}^M - \frac{m_x}{m_{H_2O}}(\nu_{H_2O}{}^P - \nu_{H_2O}{}^M) \quad (7)$$

where the superscripts P and M refer to tubulin subunits in the polymer and monomer states, respectively. At low ligand concentrations, $m_x < 10^{-2}$, the last term becomes negligibly small, since $m_x/m_{H_2O} = <10^{-2}/55.5 = <1.8 \times 10^{-4}$, and d ln K_p/d ln a_x can be equated within a close approximation with $\Delta\bar{\nu}_x$, the difference between the number of ligand molecules bound[3] to a tubulin subunit in the polymer and monomer states.

The results of such experiments carried out as a function of magnesium concentration, between 1 and 2×10^{-2} M $MgCl_2$ total concentration, are shown in Figure 5 and Table IV.[4] From the straight line obtained, it is obvious that, within the magnesium concentration range studied, the formation of microtubules is favored by an increase in Mg^{2+} ion concentration. This is in contrast to the report of Olmsted and Borisy (1975), who reported that magnesium concentrations greater than 1×10^{-3} M inhibited microtubule self-assembly from cycle-

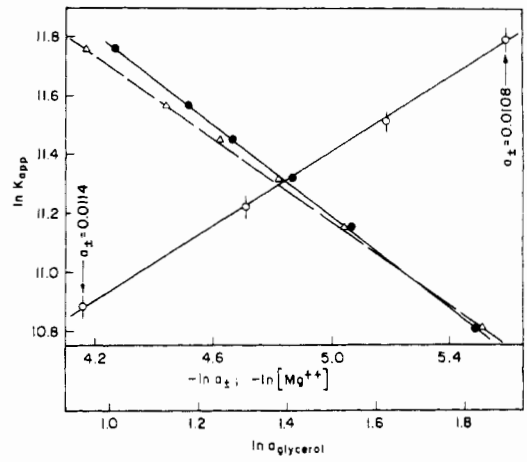

FIGURE 5: Dependence of the apparent propagation constant on $MgCl_2$ and glycerol. Filled circles: ln K_{app} as a function of $MgCl_2$ activity ($a_\pm$); triangles: ln K_{app} function of free magnesium ion concentration; open circles: ln K_{app} as a function of glycerol activity; the values of the calculated $MgCl_2$ activity at the two extreme glycerol concentrations are marked on the figure. The solvent was the phosphate assembly buffer at pH 7.0 and 37 °C; the experiments as a function of $MgCl_2$ were done in 3.4 M glycerol; the experiments as a function of glycerol were done in 1.6×10^{-2} M $MgCl_2$.

prepared tubulin. The slope of the straight-line plot according to eq 4 gives $\Delta\bar{\nu}_{pref} = 0.78$.

Results of similar experiments on the effects of hydrogen ions, calcium, and GTP are shown in Figures 6 and 7. It is evident that increase in hydrogen ion activity between pH 6.33 and 7.00 enhances the self-association. In the pH-dependence experiments, the total $MgCl_2$ concentration was kept constant at 0.016 M, resulting in a variation[4] of the mean ionic activity, $a_\pm$, from 0.0110 at pH 7.00 to 0.0125 at pH 6.30. The values of ln K_{app} were, therefore, all corrected to $a_\pm = 0.0110$ prior to plotting, the largest correction amounting to 0.09, or within experimental error. The resulting straight-line plot (Figure 6) has a slope of 0.86 ± 0.15, indicating that one additional proton is being apparently bound to protein for each subunit added to the growing structure. The data for calcium and GTP are plotted as a function of the constituent concentration of the ligand, rather than of the mean ionic activity of the free ligand, since neither the stoichiometry nor the free energy of binding of these ligands to tubulin is known, and, therefore, a correction for bound ligand is not possible. The results show that microtubule formation is inhibited by calcium at concentrations greater than 3×10^{-5} M. At 1.2×10^{-4} M $CaCl_2$, the critical concentration reaches a value of 5.1 mg/mL. The effect of lead was also examined. It was found that at lead acetate concentrations up to 1×10^{-4} M, the effect on microtubule reconstitution was weak; at 1×10^{-4} M $PbAc_2$, $K_p = 0.7 \times 10^5$ L/mol, whereas, in the absence of lead, the value was 1.2×10^5. It was not possible to pursue this examination to higher lead concentrations, because of problems caused by hydrolysis and precipitation.

The requirement for GTP was carefully examined at ligand concentrations below 1×10^{-4} M. When GTP was absent, heating of tubulin solutions resulted in an irreversible increase in turbidity (Lee and Timasheff, 1975). Electron microscopic examination, shown on Figure 3C, revealed the presence only of rings as regularly structured assemblies, in addition to amorphous aggregates. No microtubules were seen. In the presence of GTP, heating led to a fully reversible increase in turbidity. The data, analyzed in terms of K_p, are shown on Figure 7. The propagation constant is found to increase sharply

[3] The term binding in this context means strictly a measure of thermodynamic interactions, without any assumptions about specificity, stoichiometry, mechanism, or, indeed, contact between the protein and the ligand.

[4] The concentration of free magnesium ions in solution, used in the figure and in the subsequent calculations, was obtained by correcting for the formation of $MgHPO_4$, using ionization constants measured by Tabor and Hastings (1943) and by Greenwald et al. (1940). Similarly a correction was made for the magnesium bound to tubulin, using the data of Frigon and Timasheff (1975b). This correction was of the order of 3×10^{-4} M and did not affect the results significantly.

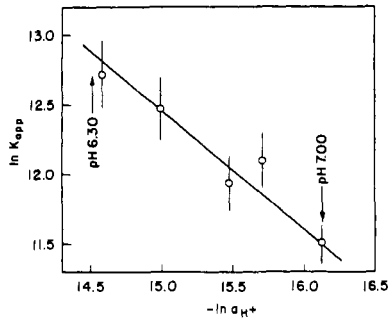

FIGURE 6: Dependence of the apparent propagation constant on hydrogen ion concentration. The solvent was the phosphate assembly buffer with H^+ as the variable at 37 °C.

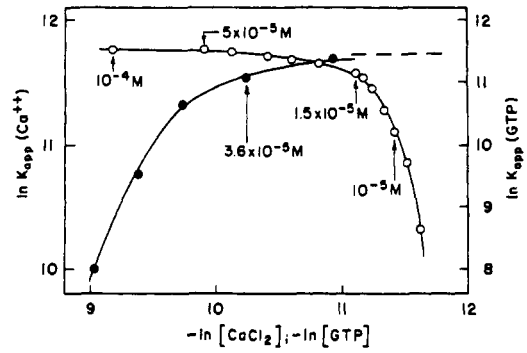

FIGURE 7: Dependence of the apparent propagation constant on $CaCl_2$ and GTP. Filled circles: ln K_{app} as a function of total $CaCl_2$ concentration (left ordinate); open circles: ln K_{app} as a function of total GTP concentration (right ordinate). The numbers on the figure are the total ligand molarity at the given points; the dashed line is the asymptotic value of ln K_{app} in the absence of calcium. The solvent was the phosphate assembly buffer at pH 7.0 and 37 °C; it contained 10^{-3} M EGTA in the GTP experiments and no EGTA in the calcium experiments.

as the GTP concentration is raised above 1×10^{-5} M, the effect becoming saturated at GTP levels above 3×10^{-5} M. While these data indicate strong thermodynamic interaction of GTP with tubulin during microtubule propagation, no stoichiometry can be derived, since the concentration of free GTP is not known. The requirement for GTP is, nevertheless, clear. At tubulin and GTP concentrations of 1.4×10^{-5} and 1×10^{-5} M, respectively, tubulin can be induced to form microtubules once. Following depolymerization and total disappearance of turbidity by cooling at 10 °C, no turbidity could be regenerated by heating to 37 °C. Reversible turbidity was regenerated, however, by addition of GTP to this depolymerized solution to a final concentration of 10^{-4} M and heating to 37 °C, indicating that the protein had not lost its ability to polymerize. In general, when GTP was present in concentrations higher than tubulin, e.g., 3.3×10^{-5} M, the polymerization–depolymerization process could be observed for at least three cycles. On the other hand, when GTP was absent, heating in the presence of 10^{-4} M GDP did not induce any change in turbidity.

Effect of Glycerol. Glycols are known to enhance the formation of microtubules (Shelanski et al., 1973; Rebhun et al., 1975), as well as to stabilize isolated microtubules (Kane, 1962). Lee and Timasheff (1975) have shown that microtubule reconstitution from purified tubulin is favored by the presence of glycerol, although glycerol is not an absolute requirement. While the mechanism by which such compounds affect the self-assembly of microtubules is not known, it has been suggested that they act by inducing tubulin to assume a conformation which has a higher ability to polymerize or by antagonizing inhibitors of polymer formation (Rebhun et al., 1975). It was thought, therefore, of interest to examine this question. From the outset, there is one aspect of glycerol action which strikingly sets it apart from the various other ligands studied, namely, the high concentration required for activity. Glycerol, in order to be effective, must be present at concentrations between 1 and 4 M. Therefore, its activity can be neither specific nor due to strong binding to one or more sites on the protein molecule. It can act nonspecifically, however, either by inducing a required conformational change, or by general thermodynamic interaction with the protein.

Circular dichroism spectra, presented in Figure 1, show that, within the resolving power of this technique, glycerol does not induce any conformational changes in tubulin either at 23 or 37 °C. In order to gain quantitative knowledge of the effect of glycerol on tubulin self-assembly, turbidimetric measurements were carried out under polymerizing conditions and K_p was derived from the critical concentration as a function of glycerol concentration. The results, plotted in terms of eq 4, are shown in Figure 5.[5] The slope of the straight line gives a value of 0.96 ± 0.10 for $(\partial m_g/\partial m_p)^P - (\partial m_g/\partial m_p)^M$; i.e., the preferential interaction of glycerol with protein increases by one molecule of ligand per molecule of protein, when each tubulin dimer is added to a growing microtubule. Using this result, an attempt was made to estimate the expected value of K_p and, hence, C_r, in the same medium, but without glycerol. Integration of eq 4 between glycerol activities of 0 and 5.07 molal (3.4 M) gave a value of $\Delta G_{3.4M} - \Delta G_0$ between -1.8 and -1.0 kcal/mol, resulting in an estimated free energy of microtubule propagation in aqueous buffer of between -5.2 and -6.0 kcal/mol, or a critical concentration of between 6 and 13 mg/mL. This indicates that glycerol is not required for microtubule formation from purified tubulin, but that it only enhances a polymerizing capacity which is intrinsically present within the protein. Subsequent measurements of the critical concentration in a medium consisting of 0.01 M, pH 7.0, phosphate buffer, 1×10^{-4} M GTP, 1.6×10^{-2} M $MgCl_2$ and no glycerol resulted in an experimental value of $C_r = 8 \pm 1$ mg/mL.

Discussion

The results described above clearly show that calf brain tubulin purified by the Weisenberg procedure is capable of self-assembling into microtubules in a variety of anionic, cationic, and zwitterionic buffers. The system seems to be best described as a nucleated polymerization of the type discussed by Oosawa and Kasai (1971), with the polymer propagation reaction having a considerably higher free energy than that of the self-association steps which lead to the formation of the nucleus. The data are consistent with a simple mechanism in which all 5.8S tubulin dimers are equally capable of entering into the reaction. There is no evidence of the existence of chemically distinct tubulin species, some of which are incompetent of polymerization, as has been asserted (Kirschner et al., 1974), nor is there any need to introduce the requirement for a specific protein factor which must complex with tubulin before the latter can polymerize into microtubules (Weingarten et al., 1975), nor of required preexisting nucleation centers (Borisy and Olmsted, 1972).

[5] The activity coefficient of glycerol, γ, was calculated from osmotic coefficient, ϕ, data of Scatchard et al. (1938), using the relation

$$\ln \gamma = (\phi - 1) + \int_0^m (\phi - 1) d \ln m \qquad (8)$$

By the criterion of circular dichroism, there is no difference in protein conformation between tubulin dissociated into 5.8 S, 110 000 molecular weight dimers in aqueous buffer at 20 °C, and tubulin present in the reconstitution buffer, containing 3.4 M glycerol, at 37 °C. Microtubule formation from purified tubulin requires GTP, is inhibited by calcium ions, as originally discovered by Weisenberg (1972), and is enhanced by the addition of magnesium ions.

Analysis of the effect of magnesium on microtubule propagation in terms of the Wyman linked functions relation suggests that one additional magnesium ion becomes bound per tubulin dimer as the latter is incorporated into growing microtubules, although the slope of the plot of ln K_{app} vs. ln $a_{\pm}$ is significantly smaller than unity. Plotting the same data in terms of magnesium ion concentration, rather than activity, as is frequently the practice, results in a slope of 0.71, as shown by the dashed line of Figure 5. Since these values of the slope make it difficult to distinguish between the binding of one additional magnesium ion per tubulin dimer, or per pair of tubulin dimers incorporated into a growing microtubule, it seemed desirable to subject this result to more critical examination.

In a self-association reaction, if ligand binding is an integral part of the reaction with only liganded monomer capable of entering into the polymerization reaction, the ligand binding equilibrium constant must be considered when the method of determining the association constant, K_p, involves measurements of the mass distribution of the species (Timasheff and Townend, 1968). This condition is true of the turbidimetric method for determining K_p. By definition, $K_p = C_r^{-1}$. The observed critical concentration, C_r, however, is the total concentration of all protein species both liganded and unliganded, and K_p, operationally defined in this way, is only an apparent equilibrium constant, K_{app}. According to Oosawa and Kasai (1962, 1971), the total protein concentration, C_t, is equal to

$$C_t = \frac{C_1}{(1 - K_n C_1)^2} + s\left(\frac{K_n}{K_g}\right)^2 \frac{C_1}{(1 - K_g C_1)^2} \qquad (9)$$

where C_1 is the concentration of monomer, K_n is the equilibrium constant for growth of the nucleus, i.e., for each step of the linear polymerization process which leads to the formation of the nucleus, n is the degree of polymerization of the nucleus, $RT \ln s = -\Delta G^*$ is the unfavorable free energy of deforming the linear polymer into a one-turn helix, and K_g is the polymer propagation equilibrium constant. When $C_t = C_r$, C_1 becomes essentially equal to K_g^{-1}. Since, in this model, $K_n \ll K_g$, $K_n C_1 \ll 1$, and the protein at $C_t = C_r$ is essentially all in the state of monomer.

Staying within the Oosawa-Kasai model, let us consider the overall reaction, with the additional assumption that ligand binding must precede the addition of subunits to the growing helical polymer:

$$\begin{aligned} T + X &\rightleftharpoons TX;\ K_1 \\ TX + X &\rightleftharpoons TX_2;\ K_1 \\ &\vdots \\ TX_{n-1} + X &\rightleftharpoons TX_n;\ K_1 \\ TX_n + M &\rightleftharpoons MTX_n;\ K_2 \end{aligned} \qquad (10)$$

where T is tubulin dimer, X is ligand, K_1 is the ligand binding constant, and K_2 is the propagation constant for adding a liganded subunit (TX_n) to a growing microtubule M. Then

$$K_{app} = \frac{[MTX_n]}{[M]\left(\sum_{i=0}^{n} [TX_i]\right)} = \frac{K_1^n K_2 [X]^n}{\sum_{i=0}^{n} (K_1[X])^i} \qquad (11)$$

Since in the microtubule growth process each product becomes immediately the reactant for the next step in the polymerization, $[MTX_n] = [M]$, $K_{app} = (\Sigma_{i=0}^{n} [TX_i])^{-1}$. This result is identical with the conclusion drawn from the analysis of the Oosawa-Kasai model, except for the incorporation of ligand binding, and permits us to set $K_{app} = C_r^{-1}$.

Solving eq 11 for K_2, and setting $K_1 = 1.06 \times 10^2$ L/mol (Frigon and Timasheff, 1975b), a search was made for the value of n for which K_2 becomes independent of magnesium ion activity. This condition was found to be satisfied at $n = 1$, as shown in Table IV, with $K_2 = 1.85 \times 10^5$ L/mol. This corresponds to a free energy of microtubule propagation, $\Delta G_2^\circ = -7.5$ kcal/mol and a true critical concentration of 0.59 mg/mL of fully liganded tubulin at pH 7.0, in the 0.01 M phosphate buffer containing 3.4 M glycerol and 10^{-4} M GTP.[6] Therefore, the slope of 0.78 in the Wyman plot, in fact, does correspond to the binding of one additional magnesium ion during the incorporation of each tubulin subunit into a growing microtubule.

Why is there such a discrepancy between the slope of the Wyman plot and the true value of $\Delta\bar{\nu}_{pref}$? The rigorous application of the Wyman plot requires that the equilibrium constant be expressed in terms of unliganded monomer only. In a method in which all monomeric species are counted, such as that used here, the slope becomes a complex function of ligand binding and eq 4 assumes the form:

$$\frac{d \ln K_{app}}{d \ln a_x} = \Delta\bar{\nu}_{pref} - \frac{d \ln \sum_{i=0}^{n} (K_1 a_x)^i}{d \ln a_x} \qquad (12)$$

A more rigorous analysis of the system can be carried out in terms of the complete reaction scheme:

$$\begin{array}{ccc} M + T + X & \overset{K_1}{\rightleftharpoons} & M + TX \\ K_4 \updownarrow & & \updownarrow K_2 \\ MT + X & \underset{K_3}{\rightleftharpoons} & MTX \end{array} \qquad (13)$$

The apparent propagation constant is now given by:

$$K_{app} = \frac{1}{C_r} = \frac{K_1 K_2 [X]}{(1 + K_1[X])}\left(1 + \frac{1}{K_3[X]}\right) \qquad (14)$$

Setting $K_1 = 1.06 \times 10^2$; $K_2 = 1.85 \times 10^5$ and $K_4 \approx 1 \times 10^3$ (the last value was estimated from an extrapolation of K_{app} to $[Mg^{2+}] = 0$, which showed that $K_4 \ll K_2$), K_3 is found to be $\sim 2.0 \times 10^4$. For ligand concentrations of the order 10^{-2}, such as used in the present study, the term $1 + (1/K_3[X])$ on the right hand side of eq 14 becomes not much different from unity, and eq 11 is found to be a close approximation of eq 14, certainly within experimental error. From this analysis of the data, it seems reasonable to conclude that, under the conditions of the present experiments, the polymerization of purified tubulin can be accounted for best by a model which includes the incorporation of one additional magnesium ion for each step in the reaction. This, however, does not establish the direct participation of magnesium ions in the interprotein bond formed when tubulin adds to a growing microtubule. It is equally probable that the magnesium ions act indirectly, for example, through an alteration of the charge distribution on

[6] The value of ΔG°_2 must be assigned an uncertainty of ±0.3 kcal/mol, since the values of K_p at any given conditions were found to vary within these limits between different preparations of tubulin. This uncertainty, however, does not affect any of the conclusions reached, since the various effects, such as slopes of ln K_p vs. ln a, remained identical from one preparation to another.

the surface of the protein molecule, leading to the formation of a constellation of charges favorable to polymerization. Furthermore, it is not known whether the additional magnesium ion is bound before or after the polymerization step, i.e., whether the reaction is ligand mediated or ligand facilitated.

The deduced value of K_3 indicates that the magnesium ions involved in microtubule formation are bound much more strongly to tubulin in the polymer than in the monomer state ($\Delta G°$ of binding to microtubules is approximately −6 kcal/mol, while $\Delta G°$ of binding to monomeric tubulin = −3 kcal/mol). Therefore, essentially all subunits within microtubules are liganded. The same holds true for the tubulin self-association to double rings (Frigon and Timasheff, 1975a,b). In fact, a sharp increase in magnesium binding has been observed at concentrations at which the double-ring structure becomes predominant in solution (Frigon, 1974). A similar situation can be expected to exist for other ligands which enhance microtubule formation. These effects are simply direct consequences of the cooperativity between ligand binding and self-association, as described by the Wyman linked functions. In descriptive terms, these relations can be regarded as examples of the utilization of the free energy of polymerization for the enhancement of the tightness of ligand binding in the ligand-mediated polymerization, and of the reciprocal situation in the ligand-facilitated reaction. Conversely, ligands which inhibit polymerization should be bound more strongly to the monomeric species of the protein. Thus, an apparent lack of binding to polymer and absence of depolymerizing action by a ligand which inhibits polymerization, as has been reported for the effect of colchicine on microtubule formation (Wilson and Meza, 1973; Lee et al., 1974), may simply reflect a negative value of $\Delta\bar{\nu}_{pref}$ in combination with slow depolymerization kinetics. In such a system, in relations similar to eq 13, $K_3 \ll K_1$, and the ligand concentration might have to be increased by several orders of magnitude in order to produce an observable effect on the polymer.

Relations similar to eq 10–14 could be set up to treat the data on the effects of calcium and GTP. In the absence of quantitative information on the binding of these ligands, however, such an exercise does not seem warranted at present. Suffice it to say that the observed effects in themselves do not permit to make conclusions concerning the integral participation of these ligands in the association mechanism. Again, the effects may be indirect.

The effect of hydrogen ion activity appears, at first sight, amenable to analysis. The Wyman plot of Figure 6 gives a value of $\Delta\bar{\nu}_{H^+,app} = 0.86 \pm 0.15$. This suggests that the addition of each tubulin dimer to a growing microtubule is accompanied by the binding of at least one additional proton. Such proton binding could signify either the titration of a group specifically involved in the self-assembly reaction, or a general redistribution of protons bound to tubulin due to the change in the electrostatic free energy of the protein as its state changes from that of an independent 110 000 molecular weight dimer to that of an integral part of a large assembly (Timasheff, 1970). In any case, it is most unlikely that the observed binding is due to a conformational change of tubulin, since the circular dichroism spectrum of this protein remains invariant between pH 6.1 and 7.0 (Lee, J. C., Corfman, D., Frigon, R. P., and Timasheff, S. N., manuscript in preparation). A complete analysis of the pH dependence is not possible, however, since the pK of the group in question (if it is indeed a specific group) is not known. The pH range suggests an imidazole; however, it must be recalled that in β-lactoglobulin there is a carboxyl group with a pK_{app} of 7.2 (Tanford et al., 1959; Susi et al., 1959) and in lysozyme one with a pK_{app} of 6.5 (Timasheff and Rupley, 1972). The narrow pH range of tubulin stability (Gaskin et al., 1974) has precluded the extension of these studies outside of the range between pH 6.3 and 7.0.

In the present study, the microtubule propagation reaction has been found to proceed with apparent positive entropy, positive enthalpy, and negative heat-capacity changes. The values of the experimental enthalpy and entropy changes found for purified tubulin are similar in magnitude to those reported (Gaskin et al., 1974) for tubulin prepared by the cycle procedure (Shelanski et al., 1973). These values are consistent with those observed for microtubule formation in vivo (Salmon, 1975a; Stephens, 1973). They suggest a mechanism which involves the release of water molecules on polymerization, and it might be conjectured that the stabilizing forces involved are either hydrophobic or electrostatic in nature, or both (Timasheff, 1973b; Heremans et al., 1974). The known reversible depolymerization of microtubules by an increase in pressure, both in vitro and in vivo, lends further support to such a mechanism. Indeed, the binding of magnesium ions which accompanies polymerization is consistent with the involvement of electrostatic interactions. Furthermore, it seems of interest that a different type of magnesium-induced self-association of tubulin, leading to the formation of closed-ring structures, is known to be impeded by an increase in pressure (Frigon and Timasheff, 1975b). There is no reason to believe, however, that this last reaction is in any way related to microtubule formation.

The values of the thermodynamic parameters, reported in this paper, are all based on the Oosawa–Kasai relation, $K_p = C_r^{-1}$. Therefore, the reported enthalpy, calculated by the van't Hoff relation, ΔH_{app}, is operationally defined as

$$\Delta H_{app} = -R \frac{d \ln K_p}{d(1/T)} = R \frac{d \ln C_r}{d(1/T)} \tag{15}$$

Returning to considerations of the polymerization theory of Oosawa and Kasai (1971), the critical concentration, C_r, can be expressed as

$$C_r = K_g^{-1} \left(\frac{K_g - K_n}{K_g}\right)^{-2} \left[1 + s \left(\frac{K_n}{K_g}\right)^{n-1} \left(1 - \frac{K_n}{K_g}\right)^2\right] \tag{16}$$

where the symbols have their previously defined meaning. In their analysis of such a polymerizing system, Oosawa and Kasai (1971) have estimated reasonable values for the various free energy relationships; $K_n/K_g = 2.4 \times 10^{-3}$, since the number of contacts per monomer added is greater in the helical polymer than in the linear one, and $\Delta G^* = 5$ kcal/mol. Using even smaller values for these parameters, $\Delta G^* = 2.5$ kcal/mol and $K_n/K_g = 10^{-2}$, the term in brackets on the right-hand side of eq 16 reduces to unity, since n is a large number (a value close to 13 would seem intuitively reasonable for the nucleation of a microtubule). Even setting $s = 1$ does not change the situation. Combination of the reduced eq 16 with eq 15 results in

$$\Delta H_{app} = -\Delta H_g + 2R \frac{d \ln (K_g - K_n)}{d(1/T)} \tag{17}$$

Since within the model used, $K_n < 10^{-2} K_g$, eq 17 reduces within a very close approximation to $\Delta H_{app} = \Delta H_g$; i.e., the critical concentration method yields essentially the enthalpy change of the helix growth reaction.

Recalling that the present method of determining K_p is

based on measurements of the mass distribution of species, and that the binding of one magnesium ion appears to be involved in the polymerization, combination of eq 11 or 14 with eq 15 and 17 gives for ΔH_2, the enthalpy of addition of a tubulin dimer to the growing microtubule:

$$\Delta H_2 = \Delta H_{app} - \frac{\Delta H_1}{(1 + K_1[Mg])} \quad (18)$$

where ΔH_1 is the enthalpy of magnesium binding to tubulin.

The heat capacity change, $\Delta C_p = d\Delta H/dT$, is then found to be

$$\Delta C_{p,2} = \Delta C_{p,app} - \frac{\Delta C_{p,1}}{(1 + K_1[Mg])} + \left(\frac{\Delta H_1^2}{RT^2}\right)\left(\frac{K_1[Mg]}{(1 + K_1[Mg])^2}\right) \quad (19)$$

where $\Delta C_{p,1}$ and $\Delta C_{p,2}$ are the heat capacities of magnesium binding and of microtubule growth, respectively.

Even though at present the values of ΔH_1 and $\Delta C_{p,1}$ for magnesium binding to tubulin are not known, it seems worthwhile to estimate the contributions of magnesium binding to the measured thermodynamic parameters listed in Table III. Assuming a value of $\Delta H_1 = 5$ kcal/mol, which is in the range observed for metal binding to proteins (Brewer, 1974; Hunt et al., 1972; Henkens et al., 1969) as well as of the enthalpy for chelation of Mg^{2+} by EDTA and EGTA (Anderegg, 1964), it is found that, for 1.6×10^{-2} M $MgCl_2$, the enthalpy values of Table III should be reduced by approximately 2.5 kcal/mol to give ΔH_2. The heat-capacity change, $\Delta C_{p,2}$, would have a value of $\sim(-1450 + 0.75\ \Delta C_{p,1})$ cal/deg-mol. It appears, therefore, that, even after correction for the binding of the metal ions, the enthalpy, unitary entropy, and heat-capacity changes associated with the addition of each tubulin dimer to a growing microtubule will remain consistent with the in vivo values of these parameters and with a mechanism that involves the release of ordered water molecules during polymerization. Negative values of ΔH_1 would result in even more positive values of ΔH_2 and ΔS_2.

Turning next to the question: why does glycerol enhance the self-assembly of microtubules? The experimental observation is that the increase in K_{app} is accompanied by a change in the preferential interaction between protein and solvent components, $\Delta\bar{\nu}_{pref} = +1$ molecule of glycerol per molecule of tubulin dimer added to the growing structure. This value of $\Delta\bar{\nu}_{pref}$ remains identical for K_2. In terms of eq 4 and 5, this means that, on polymerization, the solvent composition in the immediate domain of the protein becomes enriched in glycerol relative to bulk solvent composition, as the protein monomer is incorporated into the polymer structure. Such an enrichment need not mean that one molecule of glycerol becomes bound to protein (in the sense of complex formation) in the course of polymerization. To the contrary, it is most unlikely that the observed effect of glycerol operates through the complexing of one glycerol molecule with tubulin. Glycerol, in order to be effective, must be present at high concentrations, 1–4 M; namely, the macromolecules are situated in an environment which is 10–30% glycerol by volume. Such a concentration requirement would be consistent with a binding constant of $\sim 10°$ L/mol, with a free energy of binding of 0 kcal/mol. Had specific binding of glycerol to protein been involved, the enhancement of the self-assembly should have been evident at considerably lower glycerol concentrations. The explanation must, therefore, be sought elsewhere. Let us examine eq 5. We find that the only requirement for $\Delta\bar{\nu}_{pref}$ to be positive is that $(\partial m_x/\partial m_p)^P$ should be more positive than $(\partial m_x/\partial m_p)^M$. This situation may be obtained whether both quantities are positive or negative. It has been shown (Timasheff et al., 1976b; Gekko, K., and Timasheff, S. N., manuscript in preparation) for a number of proteins that, in aqueous glycerol medium, $\partial m_x/\partial m_p$ is negative. The proteins are preferentially hydrated, or glycerol is preferentially excluded from the immediate domain of the protein. Therefore, $\Delta\bar{\nu}_{pref}$ will be positive during tubulin polymerization simply if this process is accompanied by a decrease in preferential exclusion of glycerol from the domain of the protein.

Preferential interaction is most frequently expressed in the notation of binding; yet, it is strictly a measure of the effect of solution components on their respective chemical potentials, since

$$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,P,\mu_3} = -\left(\frac{\partial \mu_3}{\partial m_2}\right)_{T,P,m_3} \Big/ \left(\frac{\partial \mu_3}{\partial m_3}\right)_{T,P,m_2} \quad (20)$$

where component 2 is protein and component 3 is glycerol; μ_i is the chemical potential of component i, $\mu_i = \mu°_i(T,P) + RT \ln m_i + RT \ln \gamma_i$, and γ_i is the activity coefficient of component i. Therefore,

$$\Delta\left(\frac{\partial \mu_3}{\partial m_2}\right)_{T,P,\mu_3} = \Delta\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,\mu_3} = -RT\Delta\bar{\nu}_{pref}\left(\frac{1}{m_3} + \frac{\partial \ln \gamma_3}{\partial m_3}\right) \quad (21)$$

Equations 20 and 21, show that a negative value of $(\partial m_3/\partial m_2)_{T,P,\mu_3}$ means that the introduction of protein and glycerol into the presence of each other leads to an increase in the chemical potentials of both, the system becoming thermodynamically less favorable. A positive value of $\Delta\bar{\nu}_{pref}$, on the other hand, means that $(\partial\mu_3/\partial m_2)_{T,P,\mu_3}{}^{products}$ is less positive than $(\partial\mu_3/\partial m_2)_{T,P,\mu_3}{}^{reactants}$; i.e., the thermodynamic instability of the system is reduced by a displacement of the equilibrium to the right. What are the ways in which the system may relieve the thermodynamic instability brought about by the introduction of glycerol? It could do so by inducing a change in protein conformation to one in which contact with glycerol is less unfavorable, or by reducing the degree of contact between the protein and the unfavorable solvent component. In the case of tubulin, no conformational change occurs; the second pathway is, however, realized by an enhancement of the self-association. Indeed, if the parameters of eq 21 become very large, this effect may lead to phase separation, as is true in the crystallization of ribonuclease by 2-methyl-2,4-pentanediol (Pittz, E. P., and Timasheff, S. N., manuscript in preparation). Thus, it appears that the effect of high concentrations of glycerol on microtubule reassembly, or indeed stability, can find a plausible explanation in terms of nonspecific general thermodynamic interactions in which molecules of the two solution components (tubulin and glycerol) need not come into contact with each other. There is no need of invoking any specific physiological action (Rebhun et al., 1975), nor does it seem warranted to speak of "glycerol catalysis" of the self-assembly, in view of the relative concentrations of the solution components (1–4 M glycerol and $\sim 10^{-5}$ M protein). Examination of these results in terms of eq 7 shows that the preferential exclusion of glycerol may be decreased by the measured amount during polymerization, if 14 water molecules leave the immediate domain of the protein subunit during the course of the reaction, while the number of glycerol molecules in that domain remains unchanged. This result, which is con-

sistent with the conclusions drawn from the observed entropy and heat-capacity changes, gives the minimal number of water molecules that can be released when tubulin-tubulin contacts are made. Any additional release of water would require the simultaneous release of glycerol, one molecule of glycerol leaving the domain of the protein for each 14 water molecules released. Whether the preferential interactions of the protein with water and accompanying preferential exclusion of glycerol is the result of strong attraction for water or of repulsion of glycerol molecules cannot be deduced from the present thermodynamic measurements. By eq 7 and 21, the net effect on tubulin self-association must be the same: addition of glycerol to the systems must increase the association constant.

One further question that must be considered is the possible effect of glycerol on the activity of other components, in particular, of magnesium ions, since the partial derivative of eq 4 and 5 requires that the activities of all other solution components be kept constant. The present experiments were done at a constant concentration of $MgCl_2$, but the mean ionic activity, $a_\pm$, varied only insignificantly over the range of glycerol concentrations used: between $a_\pm = 0.0114\ m$ in 15% glycerol and 0.0108 m in 30% glycerol. Furthermore, the lowest value of the dielectric constant of the medium was 65, or well above the threshold of ion pair formation (Singer, 1962). It can be safely concluded, therefore, that introduction of glycerol into the system had no major thermodynamic effects other than that described by preferential interactions.

Concluding Remarks. The results presented in this paper on the self-assembly of purified tubulin to form microtubules have been found to conform to a simple model, based on the Oosawa-Kasai concepts of nucleated helical polymerization, with the introduction of ligand binding into the reaction pathway. While this does not establish the model as the only possible one to the exclusion of all others, it is the simplest one that can account for all the observations, and the introduction of additional arbitrary parameters would only complicate the analysis without providing new knowledge. It is clear that all of the information can be accounted for by the simple addition of 5.8S tubulin dimers to growing microtubules, in agreement with the previous proposal of these authors (Lee and Timasheff, 1975), as well as of Weisenberg and Rosenfeld (1975), without the need of invoking intermediate structures of various geometries, such as rings, sheets, or ribbons (Kirschner and Williams, 1974; Kirschner et al., 1975; Erickson, 1974). It is true that, in the electron micrographs obtained with purified tubulin, rings are seen to be present simultaneously with microtubules (Lee and Timasheff, 1975). Does this imply that the rings are required intermediates in reassembly? The answer is no. The rings may simply be products of a parallel, independent, self-association reaction. While both reactions are stimulated by magnesium ions, there are significant differences between them. Microtubule growth is inhibited by colchicine and by very low levels of calcium. Ring formation, on the other hand, proceeds in the presence of both ligands (Tweedy, N., and Timasheff, S. N., to be published; Weisenberg and Timasheff, 1971). These differences do not exclude absolutely the possible involvement of the double rings in the pathway of microtubule assembly. The self-assembly reaction, however, can be accounted for in terms of a simpler thermodynamic model, nor is there any evidence in the literature which compellingly supports the requirement of the ring structures as intermediates in self-assembly.

Why are rings then seen simultaneously with reconstituted microtubules? Frigon and Timasheff (1975a,b) have described the thermodynamics of the magnesium-induced self-association of tubulin to double rings consisting of 26 ± 4 tubulin dimers. The free energies of addition of each dimer to a growing ring or to a growing microtubule are of the same order of magnitude, with that of microtubule growth being greater. One may, therefore, expect these two reactions to proceed simultaneously and both products to be seen. In such a case, the presence of rings and ring formation should be taken into account in the thermodynamic analysis of the reconstitution reaction (eq 11), since the experimentally observed critical concentration encompasses all species other than microtubules. The contribution of the ring formation reaction to eq 11 and 14 was estimated using equilibrium constants reported by Frigon and Timasheff, (1975b) and extrapolated to reassembly conditions. The results indicate that the value of K_2 deduced above is little affected, K_2 increasing by not more than 10%, which is well within the error of the present experiments.

Acknowledgment

The authors thank Debra Corfman and Lucy Lee for their able technical assistance.

References

Anderegg, G. (1964), *Helv. Chim. Acta 47,* 1801-1814.
Aune, K. C., Goldsmith, L. C., and Timasheff, S. N. (1971), *Biochemistry 10,* 1617-1622.
Berne, B. J. (1974), *J. Mol. Biol. 89,* 756-758.
Borisy, G. G., and Olmsted, J. B. (1972), *Science 177,* 1196-1197.
Brewer, J. M. (1974), *Arch. Biochem. Biophys. 164,* 322-325.
Cann, J. R. (1971), *Biochemistry 10,* 3713-3722.
Casassa, E. F., and Eisenberg, H. (1964), *Adv. Protein Chem. 19,* 287-395.
Engelborghs, Y., Heremans, K. A. H., De Maeyer, L. C. M., and Hoebeke, J. (1976), *Nature (London) 259,* 686-689.
Erickson, H. P. (1974), *J. Supramol. Struct. 2,* 393-411.
Frigon, R. P. (1974), Doctoral Dissertation, Brandeis University.
Frigon, R. P., and Timasheff, S. N. (1975a), Biochemistry *14,* 4559-4566.
Frigon, R. P., and Timasheff, S. N. (1975b), *Biochemistry 14,* 4567-4573.
Frigon, R. P., Valenzuela, M. S., and Timasheff, S. N. (1974), *Arch. Biochem. Biophys. 165,* 442-443.
Gaskin, F., Cantor, C. R., and Shelanski, M. L. (1974), *J. Mol. Biol. 89,* 737-758.
Glasstone, S. G. (1947), Thermodynamics for Chemists, New York, N.Y., Van Nostrand, pp 292-295.
Greenwald, I., Redish, J., and Kibrick, A. C. (1940), *J. Biol. Chem. 135,* 65-76.
Henkens, R. W., Watt, G. D., Sturtevant, J. M. (1969), *Biochemistry 8,* 1874-1878.
Heremans, K. A. H., Snauwaert, J., Vandersypen, H. A., and Van Nuland, Y. (1974), *Proc. Int. Conf. High Pressure, 4th, 1974,* p 623-626.
Hunt, J. B., Ross, P. D., and Ginsburg, A. (1972), *Biochemistry 11,* 3716-3722.
Inoue, H., and Timasheff, S. N. (1972), *Biopolymers 11,* 737-743.
Jacobs, M., Bennett, P. M., and Dickens, M. J. (1975), *Nature (London) 257,* 707-709.
Jacobs, M., Smith, H., and Taylor, E. W. (1974), *J. Mol. Biol. 89,* 455-468.
Johnson, K. A., and Borisy, G. G. (1975), in Molecules and

Cell Movement, Inoue, S., and Stephens, R. E., Ed., New York, N.Y., Raven Press, p 119–141.
Kane, R. E. (1962), *J. Cell Biol. 15,* 279–287.
Kirschner, M. W., Honig, L., and Williams, R. C. (1975), *J. Mol. Biol. 99,* 263–276.
Kirschner, M. W., and Williams, R. C. (1974), *J. Supramol. Struct. 2,* 412–428.
Kirschner, M. W., Williams, R. C., Weingarten, M., and Gerhart, J. C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1158–1163.
Lee, J. C., Frigon, R. P., and Timasheff, S. N. (1973), *J. Biol. Chem. 248,* 7253–7262.
Lee, J. C., Hirsh, J., and Timasheff, S. N. (1975), *Arch. Biochem. Biophys. 168,* 726–729.
Lee, J. C., and Timasheff, S. N. (1975), *Biochemistry 14,* 5183–5187.
Lee, Y. C., Semson, F. E., Jr., Houston, L. L., and Himes, R. H. (1974), *J. Neurobiol. 5,* 317–330.
Levi, A., Cimino, M., Mereanti, D., Chen, J. S., and Calissano, P. (1975), *Biochim. Biophys. Acta 399,* 50–60.
Marsland, D. (1970), in High Pressure Effects on Cellular Processes, Zimmerman, A. M., Ed., New York, N.Y., Academic Press, p 259.
Murphy, D. B., and Borisy, G. G. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2696–2700.
Olmsted, J. B., and Borisy, G. G. (1973), *Biochemistry 12,* 4282–4289.
Olmsted, J. B., and Borisy, G. G. (1975), *Biochemistry 14,* 2996–3005.
Olmsted, J. B., Marcum, J. M., Johnson, K. A., Allen, C., and Borisy, G. G. (1974), *J. Supramol. Struct. 2,* 429–450.
Oosawa, F., and Higashi, S. (1967), *Prog. Theor. Biol. 1,* 28–164.
Oosawa, F., and Kasai, M. (1971), *Biol. Macromol. 5,* 261–322.
Oosawa, F., and Kasai, M. (1962), *J. Mol. Biol. 4,* 10–21.
Rebhun, L. I., Jemiolo, D., Ivey, N., Mellon, M., and Nath, J. (1975), *Ann. N.Y. Acad. Sci. 253,* 362–377.
Salmon, E. D. (1975a), *J. Cell Biol. 66,* 114–127.
Salmon, E. D. (1975b), *Science 189,* 884–886.
Scatchard, G., Hamer, W. J., and Wood, S. E. (1938), *J. Am. Chem. Soc. 60,* 3061–3070.
Shelanski, M. L., Gaskin, F., and Cantor, C. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 765–768.
Singer, S. J. (1962), *Adv. Protein Chem. 17,* 1–68.
Stephens, R. E. (1973), *J. Cell Biol. 57,* 133–147.
Susi, H., Zell, T., and Timasheff, S. N. (1959), *Arch. Biochem. Biophys. 85,* 437–443.
Tabor, H., and Hastings, A. B. (1943), *J. Biol. Chem. 148,* 627–632.
Tanford, C. (1969), *J. Mol. Biol. 39,* 539–544.
Tanford, C., Bunville, L. G., and Nozaki, Y. (1959), *J. Am. Chem. Soc. 81,* 4032–4036.
Timasheff, S. N. (1970), in Biological Polyelectrolytes, Veis, A., Ed., New York, N.Y., Marcel Dekker, chapter 1.
Timasheff, S. N. (1973a), *Adv. Chem. 26,* 327–342.
Timasheff, S. N. (1973b), *Protides Biol. Fluids, Proc. Colloq. 20,* 511–519.
Timasheff, S. N., Frigon, R. P., and Lee, J. C. (1976a), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1886–1891.
Timasheff, S. N., Lee, J. C., Pittz, E. P., and Tweedy, N. (1976b), *J. Colloid Interface Sci. 55,* 658–663.
Timasheff, S. N., and Rupley, J. A. (1972), *Arch. Biochem. Biophys. 150,* 318–323.
Timasheff, S. N., and Townend, R. (1968), *Protides Biol. Fluids, Proc. Colloq. 16,* 33–40.
Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., and Kirschner, M. W. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1858–1862.
Weingarten, M. D., Suter, M. M., Littman, D. R., and Kirschner, M. W. (1974), *Biochemistry 13,* 5529–5537.
Weisenberg, R. C. (1972), *Science 177,* 1104–1105.
Weisenberg, R. C., Borisy, G. G., and Taylor, E. W. (1968), *Biochemistry 7,* 4466–4479.
Weisenberg, R. C., and Rosenfeld, A. (1975), *Ann. N.Y. Acad. Sci. 253,* 78–89.
Weisenberg, R. C., and Timasheff, S. N. (1970), *Biochemistry 9,* 4110–4116.
Wilson, L., and Meza, I. (1973), *J. Cell. Biol. 58,* 709–719.
Wyman, J. (1964), *Adv. Protein Chem. 19,* 224–285.
Zimmerman, A. M., and Marsland, D. (1964), *Exp. Cell Res. 35,* 293–302.