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Interaction of Calf Brain Tubulin with Poly(ethylene glycols)†

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ABSTRACT: The effects of poly(ethylene glycol) (PEG) on the solution properties of calf brain tubulin were investigated at pH 7.0. In vitro reconstitution is promoted by PEG with the polymers of higher molecular weight being more efficient in lowering the free energy of the propagation step of microtubule formation. The dependence of the apparent association constant of microtubule formation on PEG concentration was analyzed by the linked-function theory of Wyman [Wyman, J. (1964) *Adv. Protein Chem. 19*, 224–286], leading to a conclusion that the thermodynamic instability of the system is reduced by formation of microtubules. Such conclusion was substantiated by the results of investigation of the preferential solvent interaction of PEG with tubulin by density measurements. Application of multicomponent thermodynamic theory shows that tubulin is preferentially hydrated in all PEG solutions, leading to an increase in the chemical potential of PEG. This unfavorable thermodynamic interaction leads to phase separation as evidenced by the precipitation of tubulin at higher PEG concentrations. Concomitant monitoring of the conformation of tubulin by comparing the accessibility of sulfhydryl groups and circular dichroic spectra at pH 7.0 indicates that PEG does not induce observable structural changes in tubulin. The results of spectrophotometric titration of tyrosine residues are consistent with that of circular dichroic spectroscopic study that PEG prevents the protein from unfolding at pH 10.

The self-assembly process of tubulin to microtubules has been the subject of intensive investigation since the initial report by Weisenberg (1972) on the conditions of in vitro reconstitution of microtubules. One of the areas of major interest is the role of proteins which copurify with tubulin. Tubulin dimers (5.8 S) purified by the cycle method, followed by gel column chromatography, have been described repeatedly as being incapable of polymerization into microtubules by themselves (Kuriyama, 1975; Kirschner & Williams, 1974; Keates & Hall, 1975). Contrary to these reports, however, Timasheff

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and co-workers (Lee & Timasheff, 1975, 1977; Lee et al., 1978a) have shown that totally dissociated purified tubulin prepared by the complete, modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970; Lee et al., 1973; Frigon & Timasheff, 1975a) can be reconstituted into microtubules. Such reconstitution does not require the presence of other proteins. This observation has been confirmed by Erickson & Voter (1976), Himes et al. (1976), Weber and co-workers (Herzog & Weber, 1977; Wehland et al., 1977), and most recently by Borisy and co-workers (Murphy et al., 1977). It appears that glycerol (Lee & Timasheff, 1975, 1977) and dimethyl sulfoxide (Himes et al., 1976) at high concentrations strengthen the microtubule growth reaction, although microtubule formation can proceed in its absence (Lee & Timasheff, 1975, 1977; Himes et al., 1976; Herzog & Weber, 1977). Besides glycerol and dimethyl sulfoxide, other glycols have been shown to enhance or stabilize microtubules (Kane, 1962). Most recently, Herzog & Weber (1978) reported that homogeneous brain tubulin, free of microtubule associated proteins, forms microtubules in polymerization buffers containing dextran or poly(ethylene glycol). The enhancement of tubulin reassembly by glycerol is most likely due to nonspecific protein–solvent general thermodynamic interactions. It is, however, not certain if the other solvents which enhance microtubule formation would exert their effects by a similar mechanism.

Recently, poly(ethylene glycols) were reported to induce growth of crystals from a large variety of proteins which have not been crystallized before (McPherson, 1976). Furthermore, poly(ethylene glycols) were employed for fractional precipitation of proteins (Polson et al., 1964; Creighton et al., 1973; Cuatrecasas, 1972). In spite of the apparent usefulness of poly(ethylene glycols) to biological chemistry, the mechanism is not known. For elucidation of the mechanism of solvents which enhance microtubule formation, the effects of poly(ethylene glycol) of varying molecular weights on in vitro microtubule reconstitution and tubulin precipitation were studied by turbidity measurements and centrifugation. The effects of PEG on the structure of tubulin were probed, and the thermodynamics of protein–solvent interactions in the presence of PEG were studied. The results of such studies are reported in this paper. A preliminary report of this work has been presented earlier (Lee & Lee, 1979).

Materials and Methods

PEG[1] 1000, 4000, and 6000 were purchased from Fisher Scientific Co. *p*-Mercuribenzoate was obtained from Sigma Chemical Co. Extreme purity grade guanidine hydrochloride from Heico, Inc., was used without further purification other than filtration through a sintered-glass filter. Calf brain tubulin was prepared by the modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970; Lee et al., 1973; Frigon & Timasheff, 1975a). Tubulin concentrations were determined spectrophotometrically in 6 M Gdn·HCl at 275 nm with an absorptivity value of 1.03 mL/(mg cm).[2]

The formation of microtubules was followed by the turbidity method, first introduced by Gaskin et al. (1974). The assembly buffer consisted of 10^{-2} M sodium phosphate, 10^{-4} M GTP, 1.6×10^{-2} M $MgCl_2$, and 3.4 M glycerol at pH 7.0. Turbidity was measured at 350 nm on a Cary 118 recording spectrophotometer as described previously (Lee & Timasheff, 1975). The equilibrium constant for the microtubule propagation reaction, K_p^{app}, was obtained from the turbidity data as the reciprocal of the critical concentration, assuming the validity for this system of the polymerization theory of Oosawa & Kasai (1971) as demonstrated by Gaskin et al. (1974).

Tyrosine titrations were performed spectrophotometrically in a Cary Model 118 instrument. The absorption of tubulin was monitored at room temperature as a function of pH, which was measured with a Beckman Model 76 pH meter. The reference was a protein solution of identical concentration at pH 7. The intrinsic dissociation constant, pK_{int}, of the tyrosine groups can be obtained by using the Linderstrøm–Lang equation

$$\mathrm{pH} - \log \frac{\alpha}{1-\alpha} = pK_{int} - 0.868\omega\bar{Z} \equiv pK_{app} \qquad (1)$$

where $\bar{Z}$ is the average net charge of the protein at any pH, ω is the electrostatic interaction parameter, and α is the degree of ionization calculated as

$$\alpha = \Delta\epsilon_{obsd}/\Delta\epsilon_{max} \qquad (2)$$

$\Delta\epsilon_{obsd}$ and $\Delta\epsilon_{max}$ are the observed and maximal molar absorptivity changes, respectively. The increase of molar absorption coefficients of tyrosine in PEG was determined according to the methods of Herskovits (1967) and Donovan (1969), by using the tandem-cell technique.

In the calculation of pK_{int}, the values of $\bar{Z}$ are usually obtained from a full titration curve, with the assumption that no binding of salt ions occurs. Since, because of the instability of the protein, no titration curve for native tubulin could be obtained, only the apparent pK values could be determined. They were obtained by plotting log $[\alpha/(1-\alpha)]$ vs. pH. By eq 1, the intercept of this curve with log $[\alpha/(1-\alpha)] = 0$ is equal to pK_{app}.

Sulfhydryl titrations were monitored by following the formation of the mercaptide complex spectrophotometrically. Tubulin solution (1 mL) at about 0.5 mg/mL in 10^{-2} M phosphate and 10^{-4} M GTP, pH 7.0, was placed in one of the compartments of a rectangular tandem cell, and 1 mL of the PMB stock solution (4×10^{-4} M in 10^{-2} M phosphate and 10^{-4} M GTP, pH 7.0) was added to the other compartment. The reference cell was filled with a solution of 2×10^{-4} M PMB, and the spectrophotometer was adjusted to zero optical density. All measurements were made at 250 nm in a Cary 118 spectrophotometer at room temperature. After mixture of the solutions in the sample cell, the increase in absorbance due to mercaptide formation was recorded until a plateau value was reached. A value of 7.6×10^3 M^{-1} cm^{-1} was used for the extinction increment at 250 nm corresponding to the formation of the mercaptide complex (Boyer, 1954).

The conformation of tubulin was also monitored by circular dichroism using a Cary Model 60 spectropolarimeter equipped with a Model 6001 attachment. The spectra were routinely recorded from 350 to 185 nm. Overlapping spectra were obtained with 0.2- and 0.1-cm fused silica cells. A value of 109 was used for the mean residue weight of tubulin in the calculation of ellipticities, $[\Theta]$. All runs were performed at room temperature, i.e., approximately 23 °C.

Precipitation curves were obtained by mixing a tubulin solution of 0.5–2.0 mg/mL in 10^{-2} M phosphate, 10^{-4} M GTP, and 0.5×10^{-2} or 1.6×10^{-2} M $MgCl_2$ at pH 7.0 with appropriate concentrations of PEG in the same buffer at room temperature. The solution was mixed and centrifuged for 5 min at 1500*g* with a Beckman microfuge. The supernatant

[1] Abbreviations used: PMB, *p*-mercuribenzoate; Gdn·HCl, guanidine hydrochloride; PEG, poly(ethylene glycol); PEG 1000, PEG 4000, and PEG 6000, PEG with an average molecular weight of 1000, 4000, and 6000, respectively.

[2] Private communication from C. Na and S. N. Timasheff.

was then analyzed for protein spectrophotometrically at 278 nm with an absorptivity value of 1.33 mL/(mg cm) (Lee et al., 1973).

Interaction between solvent components and tubulin was monitored by density measurements using previously published procedures (Lee & Timasheff, 1974). In brief, five to six solutions of tubulin at various concentrations were dialyzed against 500 mL of solvent at 4 °C for 16–24 h and at room temperature for another 4–5 h. Since the solvent consists of molecules with average molecular weights of 1000–4000, special precautions were taken to ascertain that constant chemical potential was indeed attained by dialysis. Spectrapor 4 standard cellulose dialysis tubings with molecular weight cutoff at 12000–14000 were used. Control experiments were conducted by dialyzing solutions made up in an identical manner as the protein solutions but without proteins. The densities of the solvents inside and outside of the tubing were measured after dialyzing for 16 h at 4 °C and 4 h at room temperature. Within experimental uncertainties, the density of the solution inside the dialysis tubing was identical with that of the dialysate for PEG 1000 and 4000. However, identical densities were not observed for solutions of PEG 6000 under the present experimental conditions. Protein solutions were made up by diluting a concentrated tubulin solution of about 60 mg/mL in 1 M sucrose, 10^{-2} M phosphate, 5×10^{-4} M $MgCl_2$, and 10^{-4} M GTP at pH 7.0 with the PEG solvent. The concentration of protein in each series of experiments usually ranges from 4 to 12 mg/mL. The densities of the solvents and the protein solutions were measured with a precision density meter, DMA-02D (Mettler/Paar). All measurements were made at 20 °C with the cell compartment maintained at this temperature with a refrigerated and heated circulating bath from Hotpack.

The experimental results obtained from each measurement were the densities of the solvent and of the protein solution at a given concentration. The apparent partial specific volume, ϕ, was calculated with the equation (Schachman, 1957; Casassa & Eisenberg, 1961, 1964)

$$\phi = (1/\rho_0)\left(1 - \frac{\Delta\rho}{C_2}\right) \tag{3}$$

where C_2 is the concentration of protein in grams per milliliter and $\Delta\rho$ is the difference between the densities of the solution and solvent in grams per milliliter. The calculated values of ϕ were then plotted as a function of protein concentration, and the extrapolated value was taken as the partial specific volume, $\bar{\nu}_2^\circ$.

From density measurements carried out both at constant chemical potential and constant composition of solvent components, it becomes possible to determine the extent of preferential interaction between poly(ethylene glycol) (component 3) and protein (component 2) in water (component 1), $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \equiv \xi_3$, since it is related to the change of density of the system by

$$\left(\frac{\partial \rho}{\partial C_2}\right)_{T,\mu_1,\mu_3} = (1 - \phi_2'\rho_0)^\circ = (1 - \phi_2^*\rho_0)^\circ + \xi_3(1 - \bar{\nu}_3\rho_0) \tag{4}$$

The degree symbol indicates infinite dilution of protein; $\bar{\nu}_3$ is the partial specific volume of component 3 [poly(ethylene glycol)]; ρ_0 is the density of the solvent; ϕ_2^* is the apparent partial specific volume of the protein measured at constant molality of component 3 and is assumed to have a value identical with that of ϕ_2' in the absence of PEG in this study. ϕ_2' is the corresponding value measured at constant chemical potential. $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ may assume either a positive or negative value. A positive sign means preferential binding of component 3 to protein; a negative sign means a deficiency in component 3 in the immediate domain of the protein and, thus, preferentially interacting with water, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, which is given by

$$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = -\frac{1}{g_3}(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \tag{5}$$

where g_3 is the solvent composition in grams of component 3 per gram of water.

Knowing the preferential interaction parameter $(\partial g_3/\partial g_2)$, it is possible to calculate the absolute solvation (total amount of component 3 bound to protein) or the absolute hydration of the protein (Inoue & Timasheff, 1972), since

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = A_3 - g_3A_1 \tag{6}$$

where A_3 is the absolute solvation, A_1 is the absolute hydration, and g_3 is the solvent composition. Equation 6 can be rewritten as (Reisler et al., 1977; Kupke, 1973)

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = A_3 - E_3 - g_3A_1 \tag{7}$$

where E_3 is the grams of component 3 excluded per gram of component 2. If one assumes A_1, A_3, and E_3 are independent of solvent composition, an assumption not supported by any evidence, it is possible to obtain values for A_1 and $A_3 - E_3$ by eq 7.

Preferential interaction has been shown to be simply a re-

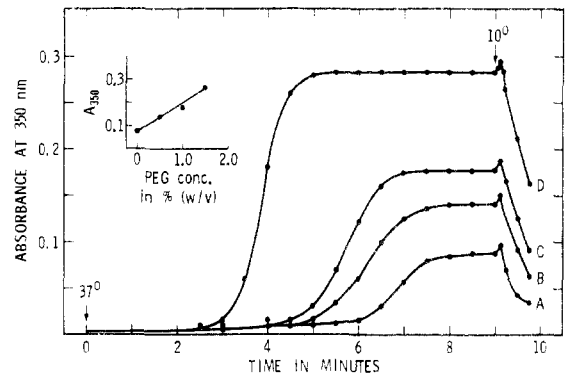

FIGURE 1: Turbidimetric measurements of tubulin in the presence of PEG 1000. The conditions are as follows: 10^{-2} M sodium phosphate, 10^{-4} M GTP, 1.6×10^{-2} M $MgCl_2$, and 3.4 M glycerol, at pH 7.0 and 37 °C. Tubulin concentration in all cases is 2.1 mg/mL. The final concentrations of PEG 1000 are as follows: (A) none; (B) 0.5% (w/v); (C) 1.0% (w/v); (D) 1.5% (w/v).

Table I: Dependence of Microtubule Growth on Poly(ethylene glycol) Concentration

[poly(ethylene glycol)] M	g/100 mL	K_{app} (L/mol)	$\Delta\bar{\nu}_{pref}$
PEG 1000			
0.50×10^{-2}	0.50	1.76×10^5	
0.75×10^{-2}	0.75	2.50×10^5	
1.00×10^{-2}	1.00	3.21×10^5	
1.50×10^{-2}	1.50	5.40×10^5	1.02
PEG 4000			
0.75×10^{-3}	0.30	2.01×10^5	
1.25×10^{-3}	0.50	3.00×10^5	
1.50×10^{-3}	0.60	3.31×10^5	
2.25×10^{-3}	0.90	4.38×10^5	0.71
PEG 6000			
0.50×10^{-3}	0.30	3.00×10^5	
0.83×10^{-3}	0.50	3.48×10^5	
1.30×10^{-3}	0.78	4.79×10^5	
1.60×10^{-3}	0.96	4.79×10^5	0.50

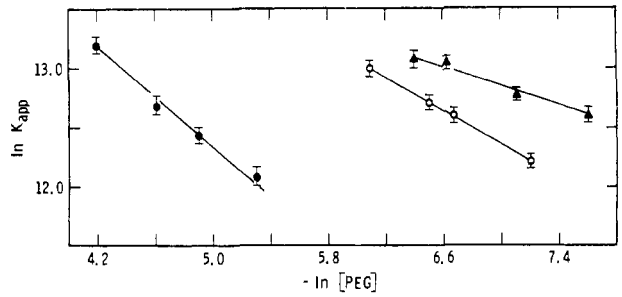

FIGURE 2: Dependence of the apparent propagation constant on PEG concentrations. The symbols and identity of PEG are as follows: (●) PEG 1000; (○) PEG 4000; (▲) PEG 6000. The solvent was the phosphate assembly buffer at pH 7.0 and 37 °C.

flection of the perturbation of the chemical potential or activity coefficient of component 3 by addition of the macromolecule, which may be expressed in terms of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ since (Timasheff, 1973; Lee et al., 1975; Pittz & Timasheff, 1978)

$$(\partial m_3/\partial m_2)_{T,p,\mu_3} = -(\partial \mu_3/\partial m_2)_{T,p,m_3}/(\partial \mu_3/\partial m_3)_{T,p,m_2} \quad (8)$$

where

$$\partial \mu_3/\partial m_3 = \frac{RT}{m_3} + RT\frac{\partial \ln \gamma_3}{\partial m_3}$$

and

$$\partial \mu_2/\partial m_3 = \partial \mu_3/\partial m_2 = -(\partial g_3/\partial g_2)\frac{M_2}{M_3}RT\left(\frac{1}{m_3} + \frac{\partial \ln \gamma_3}{\partial m_3}\right)$$

and M_i is molecular weight.

Results

The effect of poly(ethylene glycol) on in vitro microtubule reconstitution was studied by the turbidimetric method of Gaskin et al. (1974). The results of such experiments in the presence of PEG 1000 are shown in Figure 1. It was found that the turbidity values in the plateau regions are proportional to the PEG concentrations, as shown in the inset of Figure 1. Furthermore, the turbidity generated by heating at 37 °C can be reversed by lowering the temperature to 10 °C, as shown in Figure 1, and also is sensitive to Ca^{2+} ions. These are properties attributed to microtubules (Olmsted & Borisy, 1973). The turbidity monitored, therefore, is most likely due to the formation of microtubules, the reconstitution of which is favored by PEG 1000. For quantitation of the effect of PEG on the thermodynamics of microtubule reconstitution, the perturbation of PEG in the apparent microtubule association constant, K_{app}, was monitored by turbidity measurements. The results, presented in Table I, show that the presence of PEG increases the association constant and the increase is a function of PEG concentration. Another interesting observation is that in order to enhance the K_{app} to about 3×10^5 L/mol the concentration ratio of PEG 1000/PEG 4000/PEG 6000 is approximately 18:2.7:1. Such results indicate that the increase in association constant is a function of the size of PEG. The larger the PEG molecule, the more effective it is in enhancing the reconstitution of microtubule.

The data were further analyzed in terms of the linked-function relations developed by Wyman (1964) and extended by Tanford (1969) and by Aune et al. (1971). It has been shown (Lee & Timasheff, 1977; Pittz & Timasheff, 1978) that for any reaction which depends on a solvent variable, at constant temperature, pressure, and activity of all other solution components, the equilibrium constant, K, will vary with solvent composition as

$$\frac{d \ln K}{d \ln a_X} = \Delta\bar{\nu}_{pref} - \frac{n_X}{n_{H_2O}}\Delta\bar{\nu}_{H_2O} \quad (9)$$

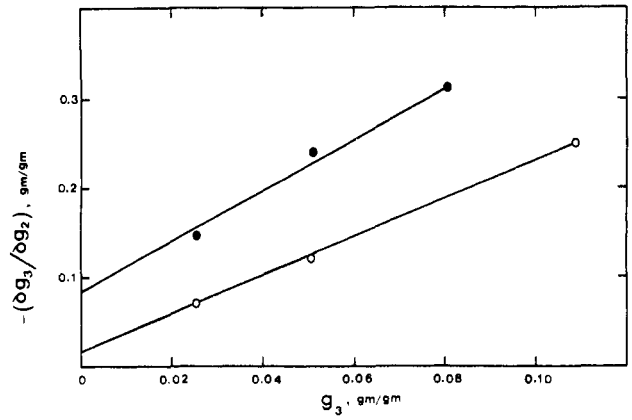

FIGURE 3: Relationship between the preferential solvent interaction parameter, $\partial g_3/\partial g_2$, and solvent composition, g_3. Open circles represent data for PEG 1000 and filled circles represent data for PEG 4000.

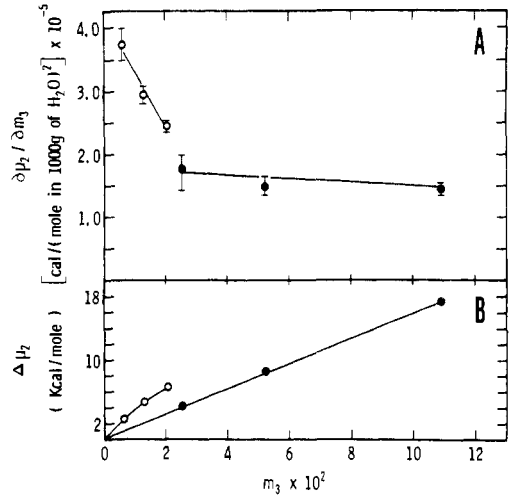

FIGURE 4: Effect of PEG on the chemical potential of tubulin at pH 7.0 and 20 °C. (A) $\partial\mu_2/\partial m_3$ as a function of PEG molal concentration. (B) $\Delta\mu_2$ as a function of PEG molal concentration. The symbols and identity of PEG are as follows: (●) PEG 1000; (○) PEG 4000.

where $\Delta\bar{\nu}_{pref}$ and $\Delta\bar{\nu}_{H_2O}$ are the differences between the preferential interaction of solvent components and water, respectively, with the two end states of the reaction in question and n_X/n_{H_2O} is the ratio of the number of moles of component X to that of water present in the system. The second term on the right will be negligible; therefore, it is simplified to $\Delta\bar{\nu}_{pref}$. In standard multicomponent thermodynamic notation (Casassa & Eisenberg, 1964; Timasheff, 1973), this parameter can be expressed as

$$\Delta\bar{\nu}_{pref} = \left(\frac{\partial m_3}{\partial m_2}\right)_{\mu_3}^{polymer} - \left(\frac{\partial m_3}{\partial m_2}\right)_{\mu_3}^{monomer} \quad (10)$$

where m_i is concentration in molal units. The results of analysis in accordance to eq 9 are shown in Figure 2. Since the activity coefficient of PEG is unknown, the results are plotted in terms of ln K_{app} vs. ln PEG concentration. In all cases straight line plots were obtained. Values of $\Delta\bar{\nu}_{pref}$ for the corresponding PEG are presented in Table I. Positive values are obtained for all three polymers tested. In terms of eq 9 and 10, this means that, on polymerization, the solvent composition in the immediate domain of the protein becomes enriched in PEG relative to bulk solvent composition, as the protein monomer is incorporated into the polymer structure. Such an enrichment does not necessarily mean that PEG becomes bound to tubulin during polymerization. A positive value for $\Delta\bar{\nu}_{pref}$ is achieved when $(\partial m_3/\partial m_2)^{polymer}$ is more

Table II: Preferential Interactions of Calf Brain Tubulin with Solvent Components in Water–Poly(ethylene glycol) Systems at pH 7.0

% PEG (g/100 mL of solution)	ϕ_2' (mL/g)	ϕ_2^{*a} (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_1/\partial m_2)_{T,\mu_1,\mu_3}$ $\times 10^{-4}$ (mol/mol)	$(\partial\mu_3/\partial m_2)_{T,p,\mu_3}$ $\times 10^{-5}$ [cal/(mol in 1000 g of H_2O)^2]
PEG 1000							
0	0.736 ± 0.002						
2.5	0.747 ± 0.002	0.736	−0.07 ± 0.01	−8 ± 1	2.7 ± 0.3	1.7 ± 0.1	1.8 ± 0.3
5.0	0.755 ± 0.002	0.736	−0.12 ± 0.01	−13 ± 1	2.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.1
10.0	0.773 ± 0.002	0.736	−0.25 ± 0.01	−28 ± 1	2.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
PEG 4000							
2.5	0.760 ± 0.002	0.736	−0.15 ± 0.01	−4.1 ± 0.3	5.8 ± 0.2	3.5 ± 0.2	3.8 ± 0.2
5.0	0.774 ± 0.002	0.736	−0.24 ± 0.01	−6.6 ± 0.3	4.6 ± 0.1	2.8 ± 0.1	3.0 ± 0.2
10.0	0.783 ± 0.002	0.736	−0.31 ± 0.01	−8.5 ± 0.3	3.8 ± 0.1	2.3 ± 0.1	2.5 ± 0.1

[a] These numbers are not experimentally determined. It is assumed that ϕ_2^* has a value identical with that of ϕ_2' in the absence of PEG, which is consistent with observations in other systems.

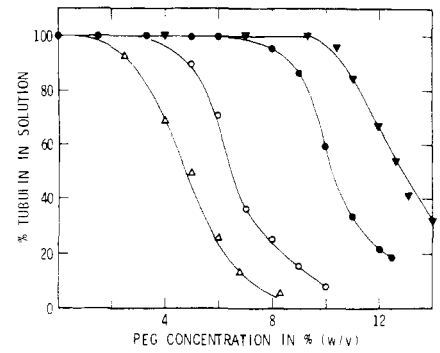

FIGURE 5: Effect of PEG on the solubility of tubulin at 22–23 °C. The solvent is 10^{-2} M sodium phosphate and 10^{-4} M GTP, pH 7.0. The final concentrations of $MgCl_2$ and the identity of PEG are as follows: (●) 1.6×10^{-2} M $MgCl_2$, PEG 1000; (○) 1.6×10^{-2} M $MgCl_2$, PEG 4000; (△) 1.6×10^{-2} M $MgCl_2$, PEG 6000; (▲) 0.5×10^{-2} M $MgCl_2$, PEG 1000.

positive than $(\partial m_3/\partial m_2)^{\text{monomer}}$. This situation may be obtained whether both quantities are positive or negative.

For determination of the magnitude of $\partial m_3/\partial m_2$, the preferential solvent interaction parameter was determined by density measurements. The results of such measurements on the system of tubulin–water–PEG at pH 7.0 are presented in Table II. At all solvent compositions, the values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, hence, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ since $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (M_2/M_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, are negative, indicating a deficiency of PEG in the domain of the protein. The extent of such deficiency increases with increasing PEG concentration as shown in column 4, Table II. Furthermore, PEG 4000 is apparently more excluded from the protein domain than PEG 1000 as indicated by the greater negative value. The data from preferential solvent interaction studies were further analyzed in accordance to eq 7, as shown in Figure 3. Linear relation between $\partial g_3/\partial g_2$ and PEG concentration was observed within the narrow range of PEG concentration studied. Values of $A_1 = 2.1 \pm 0.1$ and 2.8 ± 0.4 g/g for PEG 1000 and PEG 4000, respectively, were obtained, and the corresponding values of $A_3 - E_3$ are -0.02 ± 0.01 and -0.09 ± 0.06 g/g. Since $\partial g_3/\partial g_2$ decreases with increasing g_3, E_3 must outweigh A_3; hence, the values for $A_3 - E_3$ represent the minimum values of E_3. The exceptionally high apparent values for A_1 are consistent with the interpretation that PEG is excluded from the domain of tubulin with PEG 4000 being more so than that of PEG 1000. Although the data are internally consistent, one must view these quantitative analyses with reservation since there are no a priori reasons to accept the validity of the assumption that A_1, A_3, and E_3 are independent of solvent composition.

Preferential interaction has been shown to be simply a reflection on the change in chemical potential of solvent component in the presence of protein (Pittz & Timasheff, 1978). The values of changes in chemical potential of PEG are shown in Table II, column 8, and Figure 4. In this calculation, using eq 8, $(\partial\mu_3/\partial m_3)_{T,p,m_2}$ was approximated by RT/m_3, since data are not available on the variation of the activity coefficient of PEG concentration. The error introduced by such approximation is less than 10% of the value for RT/m_3 (Robinson & Stokes, 1959). At all solvent compositions, introduction of tubulin into the solvent system causes an increase in the chemical potential of tubulin, i.e., a destabilization of the system thermodynamically. As is evident from Figure 4, the increase in chemical potential in the PEG 4000–water system is more than that of PEG 1000, indicating a greater destabilization in the system which contains PEG of higher molecular weight.

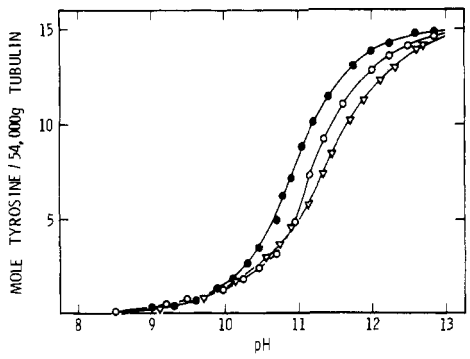

FIGURE 6: Spectrophotometric titration of calf brain tubulin in 10^{-2} M sodium phosphate at 22–23 °C. The symbols and final concentrations of PEG 4000 are as follows: (●) none; (○) 5% (w/v); (△) 10% (w/v). The protein concentration ranges from 1.1 to 1.3 mg/mL.

The lines in Figure 4A can be expressed, for PEG 1000 and PEG 4000, by

$$\partial \mu_2/\partial m_3 = 1.8 \times 10^5 - (3.3 \times 10^5)m_3 \qquad (11a)$$

and

$$\partial \mu_2/\partial m_3 = 4.2 \times 10^5 - (5.0 \times 10^6)m_3 \qquad (12a)$$

Upon integration, eq 11a and 12a can be expressed as

$$\Delta\mu_2 = (1.8 \times 10^5)m_3 - (1.7 \times 10^5)m_3^2 \qquad (11b)$$

$$\Delta\mu_2 = (4.2 \times 10^5)m_3 - (2.5 \times 10^6)m_3^2 \qquad (12b)$$

The changes in chemical potential of tubulin, $\Delta\mu_2$, as a function of PEG concentration are shown in Figure 4B. There is an increase in the chemical potential of tubulin with increasing PEG concentration, again indicating a thermodynamic destabilization of the solution in the presence of PEG.

At higher concentrations of PEG, tubulin is precipitated out of solution even at 10 °C, an observation also reported by Herzog & Weber (1978). In order to investigate the mechanism of such phenomenon, the effect of PEG concentration on the solubility of tubulin was studied. Figure 5 is a summary of the solubility of tubulin as a function of PEG 1000, PEG 4000, and PEG 6000 concentrations. At 1.6×10^{-2} M $MgCl_2$, a concentration at which formation of large aggregates is favored (Frigon & Timasheff, 1975a,b; Lee & Timasheff, 1975, 1977), precipitation can already be observed with initial protein and PEG concentrations of 0.5 mg/mL and 3–8%, respectively. The concentrations of PEG at which 50% of tubulin remains in solution are 10.2, 6.4, and 4.8% (w/v) for PEG 1000, PEG 4000, and PEG 6000, respectively. Such results indicate that the polymer with higher molecular weight is more efficient in precipitating tubulin. A similar trend was observed at 0.5×10^{-2} M $MgCl_2$ although the actual concentrations of PEG required to precipitate 50% of tubulin are higher than those at 1.6×10^{-2} M $MgCl_2$, as shown in Figure 5. It is evident that the efficiency of precipitating tubulin by PEG depends on the size of the polymer and solution variables.

The effect of PEG on in vitro microtubule assembly and precipitation of tubulin may be a manifestation of an induced structural change in the protein by PEG. The conformation of tubulin was, therefore, monitored by circular dichroism and the reactivity of tubulin sulfhydryl groups is determined. Furthermore, the apparent pK_a of tyrosine residues is also determined. The accessibility of tubulin sulfhydryl groups was investigated by reacting with PMB as a function of PEG concentration ranging from 0 to 10^{-2} M PEG 1000 and 0 to 2.5×10^{-2} M PEG 4000. The results of such studies indicated that the same number of sulfhydryl groups (8.8 ± 0.5) is titrated under all conditions. It is evident that the presence of PEG does not alter the accessibility of sulfhydryl residues

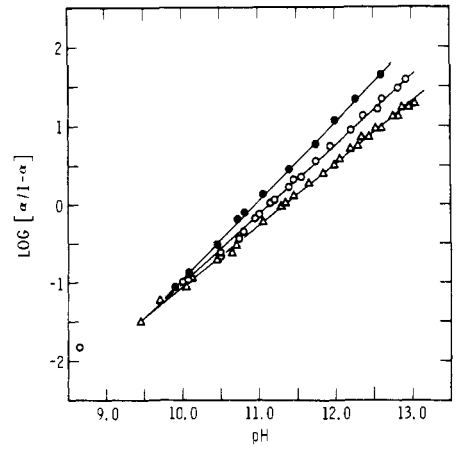

FIGURE 7: Plots of log $[\alpha/(1-\alpha)]$ vs. pH in 10^{-2} M sodium phosphate. The symbols and final concentrations of PEG 4000 are the same as those described in Figure 6. The values for pK_{app} and the slopes of these plots are as follows: (●) 10.9 ± 0.05, 1.0 ± 0.2; (○) 11.1 ± 0.05, 0.90 ± 0.02; (△) 11.3 ± 0.05, 0.78 ± 0.02.

to PMB. The reaction proceeded rapidly with 80% of the reaction completed within 30 s. The presence of PEG does not apparently bury any sulfhydryl residues; however, it might have altered the reaction rate of sulfhydryl residues with PMB but was not detected under the present experimental conditions.

The apparent pK_a of tyrosine residues was monitored as a function of PEG concentration for both PEG 1000 and PEG 4000. The results of the titration of tubulin in 10^{-2} M sodium phosphate with 0, 1.25×10^{-2}, and 2.50×10^{-2} M PEG 4000 are shown in Figure 6. The results can best be described in terms of all 15 residues per 54 000 g of tubulin ionizing between pH 9 and pH 13. The average molar extinction coefficient per tyrosine residues was found to be 2650 ± 180 in the presence or absence of PEG. Such a value is different from 2960 reported earlier by Lee et al. (1978b). The difference can be totally accounted for when the change in extinction coefficient of tubulin in Gdn·HCl is taken into consideration. In the earlier report (Lee et al., 1978b) an absorptivity of 1.15 was used, and in the present report a revised value of 1.03 is adopted. The expected extinction coefficient per tyrosine based on such a change is exactly 2650. Since the experimental uncertainties of the extinction coefficient based on nine measurements are about the same order of magnitude expected for PEG perturbation (Ingham, 1977), the value of 2650 was adopted for buffers in the presence and absence of PEG. There is an observable shift in the titration curves as shown in Figure 6. The presence of PEG apparently deters ionization of the tyrosine residues. A plot of these data in terms of the Linderstrøm–Lang equation (eq 1) is shown in Figure 7. The straight-line plot indicates that the titration proceeds essentially as that of 15 identically ionizing independent groups. The intercept of this line with log $[\alpha/(1-\alpha)] = 0$ resulted in a value of pK_{app}. It is apparent that the values of pK_{app} increased with increasing concentration of PEG 4000. The results from such titration study are summarized in Figure 7. A shift of 0.4 pH unit is observed for PEG 1000 and PEG 4000 at the highest concentration tested, i.e., 10×10^{-2} and 2.5×10^{-2} M for PEG 1000 and PEG 4000, respectively. Such a shift might be a consequence of the change in dielectric constant of the solution by the presence of PEG. Although there are no data available on the dielectric constants of PEG solutions, an attempt was made to estimate the magnitude of change in pK_{app} value that can be attributed to alterations in dielectric constant. It was assumed that PEG solutions have the same dielectric constants as methanol of the same weight concentration. It was also assumed that native tubulin at pH 10 has

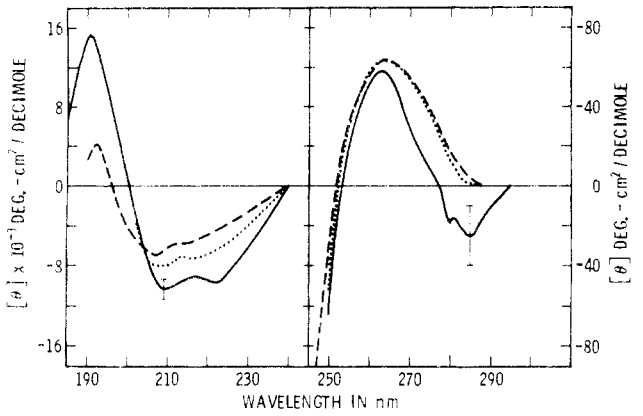

FIGURE 8: Circular dichroism spectra of calf brain tubulin in 10^{-2} M sodium phosphate and 10^{-4} M GTP at 22–23 °C as a function of pH and PEG 4000. The protein concentration ranges from 1.3 to 1.4 mg/mL. The vertical lines represent the maximum deviations observed. (—) In the presence and absence of 10% (w/v) PEG 4000 at pH 7.0; (···) in the presence of 10% (w/v) PEG 4000 at pH 10.0; (- - -) in the absence of PEG at pH 10.0.

the same number of charges as the protein in Gdn·HCl, i.e., 40 (Lee et al., 1973). It was estimated that the alteration of dielectric constant in the solution can only account for 0.14 pH unit in pK_{app}. Such a conclusion was substantiated by experimental observations that the pK_a of tyrosine solution increases by 0.1 pH unit in the presence of 10% (w/v) PEG 4000. The sum of the left-hand side of eq 1 between pH 10 and pH 12 remains constant in the absence of PEG as demonstrated by the slope of the plot in Figure 7 being 1.0 ± 0.02. It has been proposed that this indicates a small contribution of the electrostatic free energy term and that there is little electrostatic interaction between charge groups on the protein (Lee et al., 1978b). Furthermore, it was suggested that above pH 10, calf brain tubulin exists in an expanded state. The presence of PEG, however, leads to a change in the slope of these plots as shown in Figure 7. The values for the slope decrease from 1.0 to 0.78 with increasing concentration of PEG 4000 from 0 to 2.5×10^{-2} M. Similar changes were observed with PEG 1000. At 10×10^{-2} M PEG 1000, the value for the slope decreased to 0.91 ± 0.02. Such observations indicate an increasing contribution of the electrostatic free energy term with PEG present in the solution. Calf brain tubulin, therefore, most likely does not assume an expanded state under these circumstances and may actually be in a conformation which resembles more of the native form.

In order to ascertain the results from tyrosine titration experiments, the structure of tubulin was monitored by circular dichroism. If the presence of PEG 4000 does indeed prevent tubulin from assuming an expanded state at pH 10, the circular dichroism spectra should indicate the presence of a more structured conformation. Figure 8 shows the circular dichroism spectra of tubulin. The far-UV CD spectra at pH 10.0 show that in the presence of 10% (w/v) PEG 4000 tubulin does assume a conformation that is apparently more structured. Actually, it resembles the far-UV spectrum of tubulin at pH 9.0 (Lee et al., 1978b). Furthermore, at pH 7.0 the circular dichroism spectra of tubulin reveal that the presence of 10% (w/v) PEG 4000 does not alter the structure of the protein as monitored by circular dichroism. Such conclusion is consistent with the observation that the reactivities of sulfhydryl groups with PMB are not altered.

Discussion

The results from the present investigation show that the presence of PEG does not induce gross conformational changes at pH 7.0 as monitored by circular dichroism and accessibility of sulfhydryl residues to PMB. Under these conditions, the in vitro reconstitution of microtubule is favored by PEG with the higher molecular weight polymers being more efficient in enhancing microtubule formation or precipitation of the protein. Furthermore, the presence of tubulin thermodynamically destabilizes the solution by inducing an increase in the chemical potential of PEG.

Table III: Relief of Thermodynamic Destabilization of PEG–Water System

	$\Delta\bar{\nu}_{pref}$ (A)	$\partial\mu_3/\partial m_3$ (B) $\times 10^{-5}$ [cal/(mol in 1000 g of H_2O)^2]	$A \times B \times 10^{-5}$
1.0% (w/v) PEG 1000	1.02	0.58	0.6
0.5% (w/v) PEG 4000	0.71	4.66	3.3
0.3% (w/v) PEG 6000	0.50	11.65	5.9

At pH 7.0 the in vitro reconstitution of microtubule is enhanced by PEG. Analyzing the effect of PEG on the apparent equilibrium constant of the propagation step by the linked function theory derived by Wyman (1964), it was found that for all three polymers tested $\Delta\bar{\nu}_{pref}$ values are positive with values ranging from 0.50 to 1.02 (Table I). Let us examine the significance of such variations. Recalling that $(\partial m_3/\partial m_2)_{\mu_1,\mu_3,T}$ can be expressed as a change in chemical potential, one can write eq 10 as

$$\Delta\bar{\nu}_{pref} = \left[-\left(\frac{\partial\mu_3}{\partial m_2}\right)^{polymer} + n\left(\frac{\partial\mu_3}{\partial m_2}\right)^{monomer}\right] \Big/ (\partial\mu_3/\partial m_3) \quad (10a)$$

where n is the degree of polymerization. Equation 10a can then be rearranged as

$$\Delta\bar{\nu}_{pref}\left(\frac{\partial\mu_3}{\partial m_3}\right) = -\left(\frac{\partial\mu_3}{\partial m_2}\right)^{polymer} + n\left(\frac{\partial\mu_3}{\partial m_2}\right)^{monomer} \quad (10b)$$

The product $\Delta\bar{\nu}_{pref}(\partial\mu_3/\partial m_3)$ then becomes a measurement of the differences in the changes in chemical potential of PEG per monomeric unit of tubulin in the polymeric and free monomeric forms. When $(\partial\mu_3/\partial m_2)^{monomer}$ assumes a positive value and when the left-hand side of eq 10b is positive, then $(\partial\mu_3/\partial m_2)^{polymer}$ is less positive than $(\partial\mu_3/\partial m_2)^{monomer}$; i.e., equilibrium is shifted toward polymer formation to relieve the destabilization effect of tubulin on the solution. Hence, the left-hand side of eq 10b may be viewed as a measurement of the relief of thermodynamic instability of the solvent system. A more positive value indicates a greater relief; hence, the reaction toward polymer formation will be more favored. Since microtubule assembly is enhanced to about the same extent by 1.0% PEG 1000, 0.5% PEG 4000, and 0.3% PEG 6000 (Table I), one may combine the results from these experiments with those of the preferential solvent interactions (Table II) and evaluate the significance of $\Delta\bar{\nu}_{pref}$ for these polymers. For 1.0% PEG 1000, by using the values of $\Delta\bar{\nu}_{pref} = 1.02$ and $\partial\mu_3/\partial m_3 = 5.8 \times 10^4$, the left-hand side of eq 10b is 5.9×10^4. Since $(\partial\mu_3/\partial m_2)^{monomer}$ is positive (Table II), $(\partial\mu_3/\partial m_2)^{polymer}$ must be less positive than $(\partial\mu_3/\partial m_2)^{monomer}$ in accordance with the present scheme of analysis. Values for the left-hand side of eq 10b for all PEG systems tested are summarized in Table III. It is evident that the relief of thermodynamic instability of the PEG 4000 system is more than that of PEG 1000 when tubulin polymerizes into microtubules. Hence, PEG 4000 is more efficient than PEG 1000 in enhancing microtubule formation. Similarly, PEG 6000 is in turn more efficient than

Table IV: Preferential Interactions of Solvent Components with Proteins

solvent component	$\partial\mu_3/\partial g_2$ [cal/[mol (g of protein) in 1000 g of H_2O]]	ref
1 M sucrose	0.18	*a*
3.4 M glycerol	0.07	*a*
50% (v/v) 2-methyl-2,4-pentanediol	0.64	*b*
1.33 M Na_2SO_4	0.90	*a*
5% (w/v) PEG 1000	1.36	*c*
5% (w/v) PEG 4000	2.73	*c*

[a] Timasheff et al. (1976). [b] Pittz & Timasheff (1978). [c] Present work.

PEG 4000. The greater efficiency of the higher molecular weight polymers in enhancing microtubule formation is accompanied by a greater relief of destabilization of the solvent system when tubulin is reconstituted into microtubules. One should, however, view the present quantitative analysis with reservation since the solution compositions of the reconstitution and preferential solvent binding studies are not identical. In addition, the attainment of equilibrium by dialysis has only been established by density measurement. It is conceivable a true equilibrium might not have been reached especially for PEG 4000; however, the qualitative conclusions should still be valid. Nevertheless, such analysis yields a qualitative rationale for the enhancement of tubulin aggregation by the addition of PEG to the system.

Upon demonstrating that the net result of microtubule formation in the presence of PEG is a relief of thermodynamic instability in the system, one may proceed to compare the thermodynamic properties of PEG with other solvents which induce phase separation, be it precipitation or crystallization. McPherson (1976) reported that PEG can induce crystal formation of proteins which have failed to yield crystals with other conventional crystallizing solvents, e.g., $(NH_4)_2SO_4$ and 2-methyl-2,4-pentanediol. It poses the question: why is PEG apparently more efficient in inducing crystallization and do crystallizing solvents possess similar thermodynamic characteristics in the presence of proteins? A means to deduce the relative effectiveness of solvent components as precipitant might be from the increase in free energy of the system upon addition of protein. Table IV is a summary of preferential interactions of several solvent components with proteins. The solvent systems presented represent those which have been reported to stabilize proteins or induce protein crystal formation. Among these solvent systems the common characteristic which PEG shares is that these systems are all destabilized by the introduction of those proteins that were tested and under the experimental conditions employed. It is evident that PEG systems are the most destabilized: more than Na_2SO_4 and 2-methyl-2,4-pentanediol. Apparently, the ability of solvent components to stabilize or crystallize proteins might be related to the increase in free energy of these systems upon addition of protein. The more positive the value for $\partial\mu_3/\partial g_2$, the better is the solvent as a precipitant or crystallizing agent. On the basis of such rationale, then PEG 4000 should be a better crystallizing or precipitating agent than PEG 1000. The reports by Ingham (1978), Polson et al. (1964), Chun et al. (1967), and Honig & Kula (1976) and the results of the effects of PEG on solubility of tubulin presented in this report are consistent with the conclusion that PEG 4000 is a better precipitant than PEG 1000.

After establishing that the enhancement of microtubule assembly and precipitation of tubulin by PEG is the result of unfavorable interaction between PEG and proteins, it is of

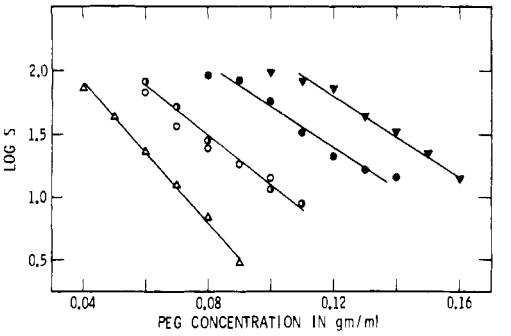

FIGURE 9: Dependence of tubulin solubility on PEG at 22–23 °C. The solvent is 10^{-2} M sodium phosphate and 10^{-4} M GTP, pH 7.0. The final concentrations of $MgCl_2$ and the identity of PEG are the same as those described in Figure 5.

interest to examine the possible molecular mechanisms which cause PEG to be excluded from the protein domain. Sinanoglu and co-workers (Sinanoglu et al., 1964; Sinanoglu & Abdulnar, 1964, 1965) have studied the solvent effects on structure of DNA molecules. The relative magnitudes of various free energy effects in different solvents were obtained. It was found that the dominant effect comes from the energy required to create a cavity in a solvent before placing a bulky solute in it. Accordingly, the interactions between the sucrose–water system and proteins were analyzed, leading to the conclusion that the structure stabilizing action of sucrose is related to its effect on the surface tension of water; i.e., addition of sucrose raises the internal pressure of the solvent, forcing the protein to overcome a greater energy barrier in unfolding (Timasheff et al., 1976). An attempt was made to correlate the results from solvent preferential interaction measurements with the change in surface tension of solvent due to change in solvent composition. PEG, however, lowers the surface tension of water.[3] Apparently, PEG may not affect the solution behavior of proteins through its influence on the surface tension of water. The current view on the partition of proteins between buffer solutions and solutions containing polysaccharides or other polymers is that proteins are excluded from a part of the volume of solution occupied by these synthetic polymers (Ogston, 1958; Ogston & Phelps, 1961; Laurent, 1963; Juckes, 1971; Ingham, 1977, 1978). Ogston and co-workers (Ogston, 1958; Ogston & Phelps, 1961) have derived a theory to relate the partition coefficient, S, of a solute to the excluded volume of the precipitant by

$$\log S = \frac{\bar{v}_3}{2.303}\left(\frac{R_3 + R_2}{R_3}\right)^3 \omega = \beta\omega \qquad (13)$$

where S = (concentration of solute in buffer)/(concentration of solute in precipitant), $\bar{v}_3$ is the partial specific volume of the precipitant, R_2 and R_3 are the effective radius of the solute and the average length of straight segments of the precipitant, and ω is the concentration of precipitant in grams per milliliter. It was also shown that

$$\frac{\text{excluded volume}}{\text{wt of precipitant}} = \frac{1}{\omega}(1 - 10^{-\beta\omega}) \qquad (14)$$

It is evident that the excluded volume is not a constant quantity per weight of precipitant (for a given solute) but varies with the concentration of the precipitant. An attempt was made to analyze the data on solubility of tubulin in the presence of PEG according to eq 13 to determine β for various PEG polymers as shown in Figure 9. Such analysis resulted in β values of 16, 20, and 28 mL/g for PEG 1000, PEG 4000, and

[3] J. C. Lee, unpublished data.

PEG 6000, respectively. The value for β apparently increases with the molecular weight of the precipitant. By substituting the values of β into eq 14 and by considering the utilization of equal concentration of precipitant, it can be shown that the most efficient precipitant of tubulin has the highest molecular weight and the largest excluded volume, and PEG 1000 being the least efficient precipitant has the smallest excluded volume. Apparently, the effectiveness of PEG in precipitating tubulin may be related to its ability to physically exclude solutes from its domain. It is unclear, however, if that is the sole molecular mechanism responsible for the solution behavior of PEG.

In conclusion, the present investigation shows that the enhancement of microtubule assembly and precipitation of tubulin by PEG is caused by an unfavorable thermodynamic interaction between tubulin and PEG leading to phase separation. This unfavorable thermodynamic interaction may be related to the physical exclusion of solute molecules by PEG; however, the possibilities of other physical properties of the solute which might also be responsible for such interaction are currently under investigation.

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