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CALORIMETRIC MEASUREMENTS OF MICROTUBULE ASSEMBLY IN VITRO

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ABSTRACT

The assembly of microtubules in extracts of mammalian brain tissue has been characterized by electron microscopy and the course of the temperature-induced polymerization of the heterodimers of α-tubulin and β-tubulin has been followed by the help of an adiabatic scanning calorimeter. Tubulin was purified by the reversible, temperature-dependent assembly procedure and was analyzed by gel electrophoresis. In a typical calorimetric experiment 1 ml of a protein solution (7.5 mg ml^{-1}) was placed into the sample cell while the same amount of this solution containing 5 mM Ca^{2+} to prevent tubulin aggregation, was placed into the reference cell. After cooling the cells to 0°C, the solutions were heated at a rate of 1 deg min^{-1} and the excess energy to keep the temperature difference of both cells close to zero was recorded as a function of temperature. The peak due to compensation of the heat of polymerization starts to rise at 20°C and declines to the base line at 32°C. The area of the peak can be calibrated in energy units by the help of an electric calibration signal. The heat of polymerization per mole of the heterodimers calculated from the experiments is 8.3 kcal. The entropy change due to this process is 27.8 e.u. The van't Hoff enthalpy calculated from the calorimetric curve is 212 kcal $mole^{-1}$ tubulin. The heat of denaturation of tubulin was determined to be 180 kcal $mole^{-1}$. The polymerization reaction was shown to be reversible whereas the denaturation is an irreversible process.

INTRODUCTION

A number of studies have been published concerning the effects of temperature [1–4] or pressure [4,5] on the in vitro polymerization of tubulin, and similar studies also have been carried out on the self-assembly of microtubules in vitro [6–12]. Thermodynamic parameters associated with polymerization, for example the van't Hoff enthalpy, can be obtained in

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such studies from plots of the log of the equilibrium constant versus the inverse of experimental temperature. The van't Hoff enthalpy can then be used to calculate the change of entropy during the reaction. However, the thermodynamic parameters obtained from a van't Hoff plot strictly hold only if the reaction studied can be described as a two-state process. In the case of microtubule polymerization, the model which describes a two-state process would be the addition of a single monomer at a time to the end of a growing microtubule. Direct, quantitative thermodynamic data, however, can be obtained only from calorimetric measurements of the reaction of interest. A disagreement between the calorimetric values and the values obtained from a van't Hoff plot rules out the possibility of a simple monomer $\rightleftharpoons$ polymer equilibrium.

Recently, a differential scanning adiabatic calorimeter [13] has been developed which makes possible the investigation of biologically relevant in vitro reactions due to its advantages of high measurement sensitivity and small sample size. At the suggestion of S. Przestalski * this instrument has been used to determine both the heat of polymerization and heat of denaturation of microtubule proteins isolated from porcine brain. The thermodynamic data thus obtained have been compared to values derived from indirect methods. The results indicate that tubulin polymerization is a cooperative process both in vitro and in vivo. We discuss various models for the in vitro polymerization of tubulin and show which are in agreement with both thermodynamic data and that derived from electron microscopy.

MATERIALS AND METHODS

Porcine brain microtubule proteins were isolated and purified by repeated cycles of assembly and disassembly using a modification of the technique of Shelanski et al. [14]. Brains from freshly sacrificed animals were minced in an equal volume of polymerization buffer (PM) consisting of 100 mM Pipes buffer [piperazine-*N*-*N*′-bis(2-ethane sulfonic acid), Sigma Chem. Co.], pH 6.95, 1 mM EDTA [di-sodium ethyleneglycol-bis-(aminoethyl ether) *N*,*N*′-(tetraacetic acid), Sigma Chem. Co.], 0.5 mM $MgSO_4 \cdot 7\ H_2O$, and 1 mM GTP [di-sodium guanosine triphosphate (PL Biochem.)], and then were homogenized at 4°C using a glass homogenizer with a motor-driven teflon pestle. The homogenate was centrifuged at 100,000 × *g* for 1 h at 4°C and an equal volume of 8 M glycerol in polymerization buffer (GPM) was added to the supernatant solution. Microtubules were then centrifuged at 100,000 × *g* for 1 h at 25°C and the pellet of microtubules was resuspended in ice cold PM (1/4 the volume of the original supernatant solution), gently homogenized by hand, and depolymerized for 30 min at 4°C. Following centrifuga-

* See Acknowledgements.

tion at 100,000 × *g* at 4°C for 50 min to remove aggregates, an equal volume of GPM was added to the supernatant solution and the solution was again incubated at 37°C for 30 min. The polymer thus formed was centrifuged, and the resulting pellet was cycled a third time through monomer to polymer. In this final cycle, the polymerization was carried out in PM without glycerol and the polymer was centrifuged at 40,000 × *g* for 30 min at 35°C in a Sorvall SS-34 rotor. Pellets were drained, frozen in liquid nitrogen, stored at −70°C and used within 3 weeks.

For a typical set of experiments, stored pellets were thawed, suspended in PM and depolymerized on ice for 30 min. The protein solution was then centrifuged at 40,000 × *g* for 30 min at 4°C to remove inactive protein and the protein concentration of the resulting supernatant solution was determined by the method of Lowry et. al. [15] using crystallized bovine serum albumin (Sigma Chem. Co.) as a protein standard. All experiments were run without glycerol in the medium. For each set of experiments, the initial polymerizability of the tubulin was assayed by centrifugation [16].

To test for protein purity, tubulin samples which had been cycled three times were run on 5% sodium dodecyl sulfate microgels in 25 mM tris-glycine buffer, pH 8.2 [17]. Gels were quantitatively stained with the dye coomasie blue and scanned at 550 nm wavelength with a Gilford model 240 spectrophotometer equipped with a linear transport.

Calorimetric measurements were made using an adiabatic differential scanning calorimeter (type DASM-1M) recently designed by P. Privalov [13] and built by the special instrumentation department of the Soviet Academy of Sciences. This instrument allows the direct determination of heat capacity differences as a function of temperature between blank and sample solutions of comparatively small volumes (1 ml) and can detect energy differences as small as 5×10^{-6} cal K^{-1} g^{-1}. The heating rate is adjustable, and can be kept constant automatically during the course of a measurement. The specific scanning rate is chosen on the basis of the kinetics of the transition that is monitored.

Prior to each calorimetric experiment, the blank and sample cells were first filled with distilled water and the calorimeter was cooled to about 0°C using an internal Peltier element. The water was then replaced by tubulin solutions. This procedure was chosen to avoid exposing the protein solutions to elevated temperatures before the actual measurement had started. The blank was filled with a tubulin solution identical to that in the sample cell, only the tubulin was incapable of polymerizing due to the addition of $CaCl_2$ to a final concentration of 5 mM [18]. The two cells were allowed to come to thermal equilibrium at 1°C. Samples containing over 3 mg ml^{-1} protein were scanned over the temperature range 5–70°C at a heating rate of 1.0° min^{-1}. A heating rate of 0.5° min^{-1} was applied but showed identical results. For protein concentrations below 3 mg ml^{-1}, however, the slower scanning rate was used. In several experiments, the heating was stopped at

37°C, and after recooling the sample to 5°C, the sample was scanned a second time to determine the degree of reversibility of the polymerization reaction.

To determine the heat of denaturation of tubulin, a sample was first scanned from 5–70°C using the buffer solution (PM) as a blank. Cooling this denatured sample to 0°C and repeating the temperature scan with the same sample probe resulted in a straight base line, indicating that the denaturation process was irreversible. The denatured tubulin sample was then used as a blank for a subsequent measurement of the heat of denaturation of a native tubulin solution of the same concentration and volume. This procedure minimized differences in the heat capacity between sample and blank solutions throughout the scan range, thereby eliminating the large, non-linear, base-line slope that otherwise was incurred.

The course of polymerization during the calorimetric experiments was assayed by monitoring a separate reaction of an identical protein solution by electron microscopy. Tubulin samples were scanned in the range 4–70°C. At three degree temperature intervals, aliquots were fixed for 1 min with isothermal fixative (2% glutaraldehyde in 50 mM caccodylate buffer, pH 7.0) to stop the polymerization reaction. The fixed samples were applied to form alvar coated grids which were glow discharged before use. The samples were then rinsed with buffer, stained with 1% uranyl acetate, air dried and examined with either a Siemens Elmiskop 1A or a Phillips 300 electron microscope operated at 80 kV.

RESULTS

Theoretical treatment of calorimetric data

The thermodynamic parameters associated with the polymerization of tubulin dimers into microtubules can be obtained from experimental determinations of the excess heat capacity, C_c, of tubulin solutions as a function of temperature.

As shown in Fig. 1, the typical excess heat capacity profile generated during tubulin polymerization is characterized by a distinct peak, which is flanked on either side by regions that are more or less linearly dependent on temperature. The temperature corresponding to the maximum of the peak, designated as T_m, is located almost exactly in the middle of the temperature-induced polymerization interval. The integral of the excess heat capacity over the temperature range of the transition is proportional to the polymerization enthalpy, ΔH. A second curve relating the fraction, θ, of the total number of subunits incorporated into microtubules during the course of polymerization is co-plotted in the same figure. This curve is obtained by plotting the fractional area under the excess heat capacity curve as a function

of temperature. The validity of this calculation depends on the assumptions that the excess heat capacity is proportional to the extent of reaction at that temperature and that the polymerization enthalpy per subunit is independent of microtubule length.

The enthalpy change, ΔH, associated with polymerization is related to the excess heat capacity and the fraction of dimers incorporated into the polymer, θ, by the equation

$$C_c = \Delta H\left(\frac{d\theta}{dT}\right). \tag{1}$$

Therefore, for the maximum value at the midpoint of the polymerization interval we can write

$$C_{c_{max}} = \Delta H\left(\frac{d\theta}{dT}\right)_{T=T_m} \tag{2}$$

If the experimental ΔH is smaller than the van't Hoff ΔH we have to assume that the polymerization is a cooperative process. For such a cooperative process [19]

$$\left(\frac{d\theta}{dT}\right)_{T=T_m} = \frac{\Delta H}{4RT_m^2}\sigma^{1/2} \tag{3}$$

or

$$C_{c_{max}} = \frac{(\Delta H)^2}{4RT_m^2}\sigma^{1/2} \tag{4}$$

For a given ΔH value then, the sharpness of the thermal transition depends on the parameter, σ, which may be considered as the probability of using a subunit to continue the growth of an existing microtubule as opposed to using it to start a new microtubule. This only holds under the assumption that nucleation is equally probable during the entire polymerization and that σ is kept constant during the polymerization. The variation of the equilibrium constant with temperature is related to the van't Hoff enthalpy, ΔH_{vH} by the equation

$$\frac{d\ln K}{dT} = \frac{\Delta H_{vH}}{RT^2} \tag{5}$$

where K is the equilibrium constant for the polymerization reaction and may be written in product/reactant form as

$$K = \frac{\theta/2}{(1-\theta)^2 c_T} \tag{6}$$

where c_T is the total concentration of interacting subunits. Equations (5) and (6) can be combined to yield the expression

$$\Delta H_{vH} = 6RT_m^2\left(\frac{d\theta}{dT}\right) \tag{7}$$

Therefore, from the slope of the θ vs. T plot at T_m, ΔH_{vH} for the polymerization reaction can be calculated according to eqn. (7). If the polymerization reaction is a simple two-state process, always involving the addition of a single subunit at a time to an existing microtubule, ΔH and ΔH_{vH} will be equal. If the two are not equivalent, their ratio gives the average number of subunits added simultaneously to an existing microtubule.

If it can be shown that the polymerization is reversible, we can apply the Gibbs–Helmholz relation

$$\Delta G = \Delta H - T\Delta S \tag{8}$$

At $T = T_m$, the midpoint of the reaction considered here, ΔG is equal to zero, since $K = 1$. Therefore, the entropy change accompanying polymerization can be calculated from the equation

$$\Delta S = \frac{\Delta H}{T_m} \tag{9}$$

Experimental data

With temperature scans over the range 4–40°C, the excess heat capacity due to polymerization was detected as a distinct peak superimposed on a base line of non-zero slope. The base line change in C_c with temperature could be eliminated for the most part by choosing as a blank, a tubulin solution whose protein concentration was identical to that in the sample cell, but which was inhibited from polymerizing by including 5 mM $CaCl_2$. A typical experimental curve using a protein concentration of 7.5 mg ml in pH buffer is shown in Fig. 1. The total enthalpy change (ΔH) is 0.5319 mcal and the heat of polymerization calculated per mole of 6S tubulin dimer of 110,000 daltons M.W. [20] is 8.3 kcal mole^{-1}. The upper and lower temperature limits for the reaction were 20 and 32°C, respectively. The maximum

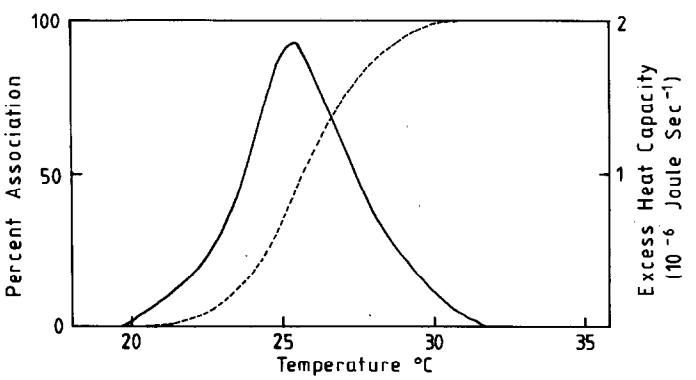


Fig. 1. Induced polymerization of a tubulin solution (7.5 mg ml^{-1} in pH buffer). ————, Differential calorimetry trace; - - - - - -, percent association. Scan rate 1° min^{-1}.

TABLE 1

Thermodynamic parameters * associated with microtubule polymerization *in vitro*

Protein concentration (mg ml^{-1})	ΔH (kcal $mole^{-1}$)	T_m (°C)	ΔS (e.u.)	ΔH_{vH} (kcal $mole^{-1}$)	$\Delta H_{vH}/\Delta H$	$\sigma \times 10^{-3}$	ΔH_{denat} (kcal $mole^{-1}$)
2.50	8.27	24.3	27.82	143.8	17.40	7.04	
5.17	7.80	26.5	26.04	172.4	22.10	4.90	175.0
5.17	8.35	26.5	27.90	162.0	21.46	4.84	172.6
7.50	8.30	25.7	26.11	212.0	25.53	3.07	180.0
9.10	8.35	27.3	27.81	206.03	24.67		
$\bar{x}$	8.21 ± 0.23	25.75 ± 1.04	27.14 ± 0.97				175.87 ± 3.78

* All parameters and their abbreviations are defined in the text.

heat capacity occurred at 25.7°C and within the margin of experimental error, T_m was in the middle of the reaction interval. Since the scanning rate in this experiment was 1.0° min^{-1}, the reaction was completed in 12 min.

The reversibility of polymerization was tested by first scanning a tubulin sample in the range 4–37°C. The scan was then stopped and the instrument was cooled again to 4°C over a time span of 90 min. For comparison, a second temperature scan was made with the same sample and blank solutions: this showed that the area under the peak was 15% less than that calculated in the first run. Therefore, it can be concluded that the polymerization reaction is almost reversible under these experimental conditions.

The effect of protein concentration on the heat capacity profile was determined by performing the measurements with solutions of tubulin ranging from 2.5 mg ml^{-1} to 9.1 mg ml^{-1}. Neither T_m nor the width of the temperature-induced polymerization interval was concentration dependent, and in all cases, the curve was symmetric.

Thermodynamic parameters associated with polymerization were calculated using the equations described in the previous section and the results are presented in Table 1 for a number of protein concentrations. Neither the calorimetric enthalpy nor entropy changes associated with polymerization are concentration dependent. The mean ΔH_{Cal} was found to be 8.21 ± 0.23 kcal $mole^{-1}$ for the 6S dimer and the mean ΔS_{Cal} was 27.14 ± 1.0 e.u. In contrast, the van't Hoff enthalpy increased with protein concentration, and in all cases, exceeded the calorimetric enthalpy. Since ΔH_{vH} is concentration dependent, the ratio of the two enthalpies also must vary with concentration. As shown in Fig. 2, this ratio, which gives the average number of subunits polymerizing at the same time, increases linearly with protein concentration up to about 7.5 mg ml^{-1}. Above this concentration, the ratio approaches a maximum value of 25 ± 1. Extrapolating the linear part of the curve to the

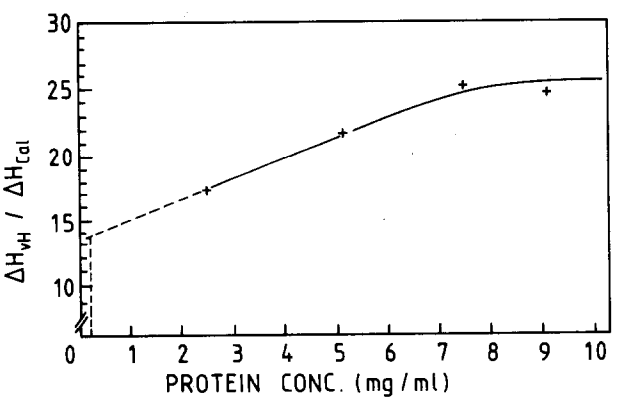


Fig. 2. A plot of the relation between protein concentration and the average number of subunits simultaneously being incorporated into polymer, as given by $\Delta H_{vH}/\Delta H_{cal}$ ratios given in Table 1.

critical concentration (0.2 mg ml^{-1}) [16] gives a vlaue of 13.83 for the number of simultaneously incorporated subunits. The experimental results in Table 1 show that the same parameter, σ, is also concentration dependent.

Figure 3 shows the differential calorimetry trace obtained in an experiment in which tubulin (5.17 mg ml^{-1} in PH buffer) was scanned over the temperature range 10–70°C. The reference solution contained denatured tubulin of the same concentration and volume. The polymerization peak between 50 and 70°C represents the combined excess heat capacity involved in both depolymerization and denaturation of microtubule proteins. When the temperature scan was continued beyond 37°C using either buffer (PM) or denatured tubulin (see Methods section) as a blank, another, much larger increase in the excess heat capacity of the solution occurred at about 60°C (Fig. 3). This peak showed a distinct shoulder on the low-temperature side in all experiments, and it is assumed that this is due to microtubule depolymerization preceding protein denaturation. This is supported by experiments in which the PM blank was replaced with a native tubulin solution of the same concentration, but containing 5 mM $CaCl_2$. In curves generated from this experiment (not shown), the shoulder appeared as a distinct peak centered at 58°C. Estimates of the area under this peak gave values that were of the same order as those due to the heat of polymerization. (However, the precise values could not be determined because the denaturation peak was not totally eradicated in these experiments.) From this fact one can conclude that the heat of denaturation is dependent on the state or history of tubulin, with less energy required to denature unpolymerized tubulin in the presence of Ca^{2+} ions.

Nevertheless, as shown in Table 1, the mean value of the combined

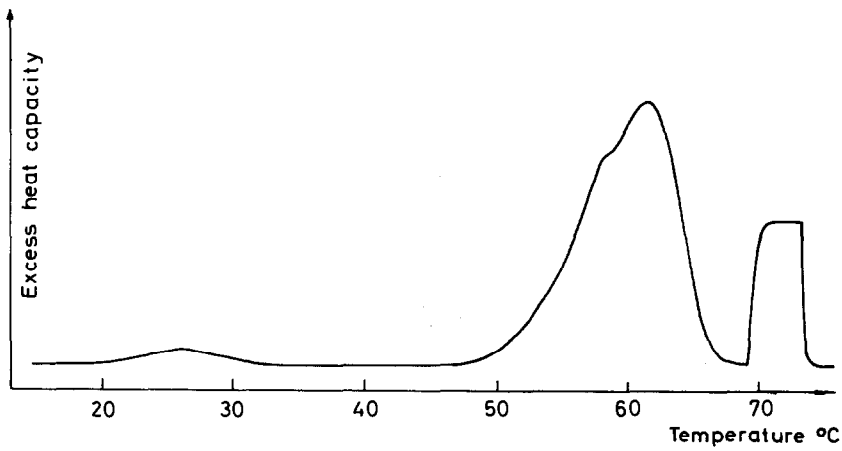
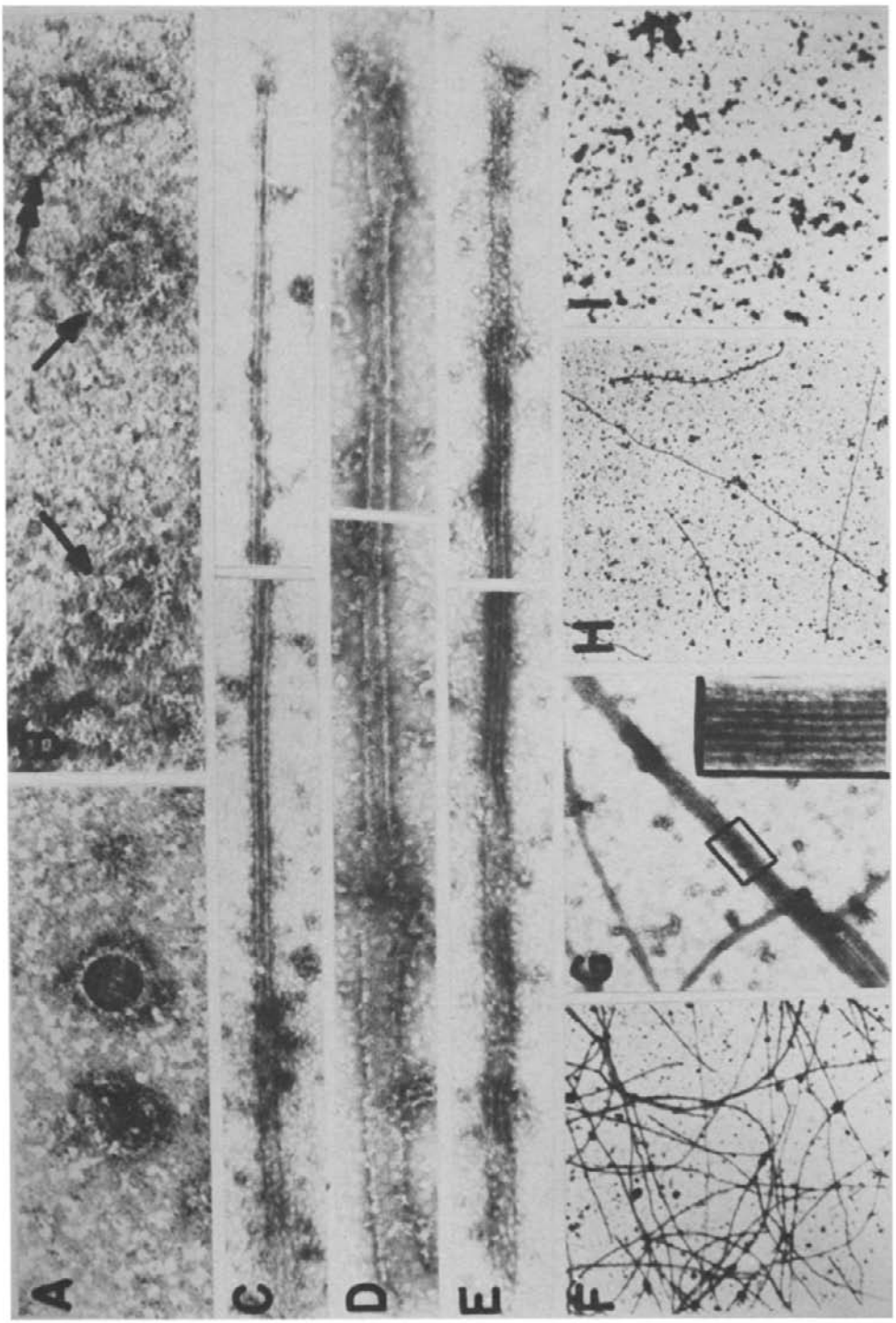


Fig. 3. Differential calorimetry trace of tubulin (5.17 mg ml^{-1} in pH buffer) scanned in the temperature range 10–70°C. The calibration peak is on the right and corresponds to 5×10^{-6} J sec^{-1}. Scanning rate 1.0° min^{-1}.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ΔH_{denat} and $\Delta H_{depolym}$ was $+175.87 \pm 3.8$ kcal mole^{-1} for the 6S dimer, and this value did not vary with protein concentration. Nor was the denaturation reaction reversible under these conditions, since a sample brought to 70°C showed only a straight line when subsequently cooled to 4°C and then scanned again to 70°C.

In order to correlate structural information from electron microscopy with the calorimetric data, separate temperature scanning experiments were performed over the temperature range 4–65°C. When the initial 4°C sample was examined, the only apparent structures were closed rings, 342 ± 1 nm in diameter (Fig. 4A). These have been shown previously to correspond to a 30S structure [16,21]. The mean circumference of these rings was 1091 ± 5.8 nm. Therefore their contour length corresponds to about 13–14 tubulin dimers, each assumed to be 80 Å long. Aliquots taken at temperatures up to 18°C showed an apparently increasing number of C-shaped structures (see →) of the same width and contour length as the closed rings (Fig. 4B). These structures appear to be derived from the rings. However, the possibility of their being formed de novo cannot be ruled out at this time. In addition to the rings and open structures, much larger, coiled structures also were present (see ↠). These structures may represent assembly intermediates composed of laterally-associated spirals (Fig. 4B).

Samples fixed at 22°C showed at least 2–3 microtubules per grid square and these possessed a typical protofilament substructure (Fig. 4C–4E). The ends of these microtubules were unfolded into flat sheets which were clearly comprised of 13 protofilaments. Microtubules derived from solutions of relatively high protein concentration (5–10 mg ml^{-1}) possessed sheets of approximately equal length on both ends (Fig. 4C). On the other hand, microtubules from solutions of lower tubulin concentration (1.0 mg ml^{-1}) either possessed sheets only on one end (Fig. 4D), or, if at both ends, the sheets were of quite different length (Fig. 4E).

Specimens fixed in the temperature range 22–35°C, which corresponds to the polymerization transition region of the heat capacity curve, showed an increasing number and length of microtubules, although no attempt was made to document these changes quantitatively (Fig. 4F). In the range of 35–43°C, there was no obvious change in either microtubule number or structure. At 48°C the staining pattern of the microtubule was much more intense and bundles of close-packed microtubules appeared as shown in Fig. 4G. In samples removed at 51°C, the numbers of microtubules were drastically reduced and non-microtubular aggregates, presumably representing

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Fig. 4. Electron micrographs of products formed during a temperature scan of 0–65°C. A; 4°C, 143,000×: B; 18°C, 141,000×: C; 22°C, 1.0 mg ml^{-1} tubulin solution, 41,600×: D; 22°C, 1.0 mg ml^{-1} tubulin solution, 105,000×: E; 22°C, 8.3 mg ml^{-1} tubulin solution, 41,600×: F; 25°C, 3,600×: G; 48°C, 24,000×: H; 51°C, 3,600×: I; 60°C, 3,600×.

denatured protein, were quite apparent (Fig. 4H). Finally, at 60°C, no microtubules were found and only the denatured aggregates were present (Fig. 4I).

DISCUSSION

The results presented in this paper show by direct thermodynamic measurements that tubulin polymerization is an endothermic process with a positive ΔH for the polymerization of 8.21 ± 0.23 kcal mole^{-1} for the 6S dimer of M.W. = 110,000 daltons. These results do not agree with those obtained in a recent calorimetric study which we became aware of after the completion of the present work [22]. There, no enthalpy change could be detected during polymerization, and it was stated that tubulin polymerization was essentially an athermic process. In addition to employing stop-flow calorimetry, Sutherland and Sturtevant [22] also used a differential scanning calorimeter identical to that used in the present study. However, they were unable to obtain reliable signals, stating that the major problem was one of obtaining proper reference solutions because of the variable amounts of glycerol remaining from tubulin isolated by the method of Shelanski et al. [14]. Because of similar inconsistent results obtained in both our study and a previous flow birefringence study [23], the tubulin used here was always passed through at least one additional monomer $\rightleftharpoons$ polymer cycle in the absence of glycerol. In addition, the protein was stored as a pellet without glycerol, and further polymerizations were carried out in buffer (PM) only. Using these procedural differences as well as tubulin solutions in both the blank and reference cells as outlined in the Methods section, we obtained consistent, reliable results at different protein concentrations as judged by the reproducibility of data obtained both from duplicate experiments and from experiments using different batches of protein.

The inability of Sutherland and Sturtevant to detect a significant enthalpy change using stop-flow methods is most likely due to a masking of the relevant heat exchange by the large exothermic peak which they incurred and showed to be unrelated to polymerization. For example, if the total 0.5 mcal of our experiment shown in Fig. 1 is divided over a 15 min reaction interval, a heat exchange rate from stop-flow calorimetry in the order of 40 μcal min^{-1} should be observed. This value, although within the sensitivity of their instrument, is quite small. In addition, the amount of polymerization, and therefore the amount of heat exchange, might have been considerably less than maximal in their experiments due to the 17°C temperature at which polymerization was carried out [2,24].

Therefore, we feel that the thermodynamic parameters obtained in the present study represent the most reliable determinations to date and we conclude that the tubulin polymerization is indeed a net endothermic pro-

cess, as has been indicated by indirect data obtained both in vitro [3] and in vivo [25].

It has often been stated that the self-assembly of tubulin and other proteins is endothermic, and therefore entropy driven [26]. This statement is an obligatory consequence of the Gibbs–Helmholz relation [eqn. (8)] where if ΔH is positive, then ΔS must also be positive. However, the entropy change of 25 e.u. calculated for the association of a tubulin dimer in this study is quite low in comparison to the van't Hoff entropy calculated both in vivo and in vitro (Table 2). This suggests that the importance of hydrophobic bonding and of the release of bound water as an entropic driving force has probably been exaggerated for the case of tubulin. Rather, other low energy interactions, such as ion pair formation, may play important roles in stabilizing the direction of dimer–dimer interactions in the intact microtubule. A role for ion pair formation is further suggested by the relatively restricted pH and ionic strength optima required for in vitro polymerization, as well as by the important role of divalent cations [18,30,31].

From a comparison of van't Hoff and calorimetric enthalpies (Table 1), one can conclude that tubulin polymerization is a cooperative process. In such a cooperative self-assembly process, one can distinguish between a nucleation step and a propagation step [32]. Furthermore, the negative heat of activation reported previously [2] suggests that the nucleation step involves the presence of some energetically unstable intermediate structures(s), which occur less frequently at elevated temperatures, slowing down the overall rate of reaction with increasing temperature.

Previous investigators have shown the presence of higher order oligomers of the 6S tubulin dimer in depolymerization products of microtubules

TABLE 2

Thermodynamic parameters measured for *in vivo* microtubule polymerization

Cell type (Oocyte)	ΔH_{vH} (kcal mole^{-1})	Cooperative unit [a]	ΔS_{vH} (e.u.)	Ref.
Chaetopterus pergamentaceous simple	36	4	124	12
equil. model nucleated condensation	7.5–22	1–3		
Pectinaria gouldii	82.2	10	286	27
Pisaster ochraceous	58.9	7	210	28
Tilia americana	33.8	4	123	29
Strongylocentrotus iroebachiensis				
grown at 0°C	64.5	8	233	8
grown at 8°C	54.9	7	197	

[a] The cooperative unit is given as the ratio of ΔH listed above to the ΔH of 8.18 kcal mole^{-1} determined for tubulin polymerization *in vitro* (Table 1).

[33–35,16]. Without these structures, polymerization either does not occur [34], or is only marginal [36]. The single ring structures found in the present study appeared to open up in the temperature range 10–20°C. This rearrangement and any others that might occur over this temperature range appear to be non-cooperative since the slope of the heat capacity curve is essentially linear in the range 0–20°C, and the 20–32°C peak lacks a shoulder on the low temperature side. In fact, the initial rise in the heat capacity curve at 20°C correlates quite well with the initial formation of microtubules which are first detected in these experiments at 18–19°C. This further suggests that the observed enthalpy change is predominately associated with the elongation process.

When the ratio of the van't Hoff and calorimetric enthalpies is extrapolated to the critical concentration of 0.2 mg ml^{-1}, defined as the minimum concentration required for polymerization to occur, the average number of tubulin dimers being incorporated into microtubules at the same time is found to be between 13 and 14. This corresponds precisely to the calculated number of subunits in the rings. However, it cannot be ruled out that these structures are involved in a separate equilibrium with the dimer before incorporation into the polymeric structure. These rings decrease in number rather early in the polymerization process [16]. The enthalpy ratio of 26 for protein concentrations above 7 mg ml^{-1} can be explained by assuming that under these conditions, the addition of dimers occurs simultaneously and equally at both ends of the growing microtubule. At intermediate protein concentrations, the average number of incorporated subunits varies between 13 and 26 (Fig. 2), suggesting an unequal growth rate at the different ends of the tubules.

The $\Delta H_{vH}/\Delta H$ ratio also rules out the possibility that polymerization could occur by addition of single dimers to sites within a microtubule [6,37], as suggested previously. In addition, incorporation of dimers at places other than the ends would require opening the microtubule lattice, a process that is energetically unfavorable and must include a molecular process to provide the required energy. However, the idea that microtubules could depolymerize by removal of dimers from sites other than the ends is compatible with other work [2,38] and cannot be ruled out from our experiments.

The values of the thermodynamic parameters obtained in the present study may be compared with thermodynamic data acquired from in vivo experiments. Inoué was the first to calculate ΔH^0 and ΔS^0 for the in vivo polymerization of microtubules by measuring the equilibrium birefringence of metaphase arrested mitotic spindles at various temperatures [6]. His calculations, as well as subsequent ones, were originally based on the assumption that a simple equilibrium exists between oriented and unoriented particles of the same molecular weight [6]. This assumption has been restated more recently by assuming the existence of a simple equilibrium between tubulin monomer and polymer, with only the oriented particles, or polymer, exhibiting birefringence [7,12].

Inoué evaluated ΔH^0 from the slope of a plot of $\ln K$ vs. $1/T$. The equilibrium constant, K, was obtained from equilibrium retardations, Γ, as a function of temperature according to $K = \Gamma/(\Gamma_{max} - \Gamma)$. The most recent determination of ΔH_{vH} for *Chaetopterus* spindles is $+36$ kcal mole^{-1}, and the accompanying entropy change derived from the Gibbs–Helmholz equation (eqn. (8)] is 124 e.u. [12]. For a simple two-state process, this enthalpy should equal the calorimetric enthalpy. Instead, it exceeds it by a factor of 4, leading to several possible interpretations.

First, if the in vivo polymerization of tubulin is really a two-state process, then ΔH, and therefore the interaction energies between dimers in vivo, must be much larger than that of brain tubulin in vitro. Comparing *Chaetopterus* mitotic apparatus microtubules for example, there must be a four-fold increase in the number of interactions between adjacent dimers, or a new class of interacting sites must come into play which contribute significantly to the dimer–dimer stability, and raise the total interaction energy four-fold.

Although small differences in the interaction energies (ΔH) will certainly occur with different environmental conditions, it is doubtful that such large differences in vivo and in vitro occur and still lead to the same complex quaternary structure of a microtubule. As shown previously, changes in solution conditions [30,39], drugs [40] and cationic substances [41,42] are capable of altering dimer interactions to such an extent that sheets, duplex microtubules and quaternary forms other than normal microtubules are formed. The most convincing evidence against large differences in binding behavior between microtubule dimers in vivo and in vitro is that the volume change that occurs upon polymerization has been calculated to be 90 ml mole^{-1} in both cases [5,12]. One would only expect this if polymerization follows the same path in vivo and in vitro.

We conclude that tubulin polymerization is most likely a cooperative process in vivo. It is possble, however, that the degree of cooperation varies from cell to cell or from state to state according to the particular intracellular conditions and the presence of various modifiers. This would provide an explanation for the fact that a number of in vivo ΔH_{vH} values reported in the literature are more or less integral multiples of the ΔH found in the present study (Table 2), and suggests that the number of tubulin subunits being incorporated at the same time in vivo varies anywhere from 2 to 8. But in no instance to date does the in vivo degree of cooperation seem to match that obtainable in vitro. This is not particularly surprising since the conditions for the in vitro polymerization of tubulin have been formulated to maximize the degree of polymerization. It is not at all unlikely that these conditions would vary somewhat in character from those present in vivo where, aside from the polymerization of tubulin, all other biochemical reactions have to function simultaneously within the same solution. It is also possible that σ is not constant under these various conditions.

The concentration of tubulin in the mitotic apparatus of sea urchin eggs

has been estimated at about 30 mg ml^{-1} [43], four times higher than in the calorimetric experiments. The degree of cooperation in vivo is at best, less than half that expected from the in vitro maximum. This suggests that the in vivo reaction is not limited by tubulin concentration. It rather looks as if elongation of microtubules in vivo is kinetically rather than energetically controlled, including some rate limiting regulatory step.

The data acquired in the present study place some restrictions on models proposed for the in vitro elongation of microtubules. The models of Johnson and Borisy [44] and Gaskin et al. [2] shown in Fig. 5A can be ruled out since they do not consider elongation to be cooperative, but rather occurring by the sequential addition of 6S dimers [44] or activated dimers [2] to the ends of a growing microtubule.

The model of Kirschner and Williams [35] (Fig. 5B) which is based on a study of microtubule depolymerization products is also unlikely to be true in its present form since it is difficult to reconcile the addition of both single subunits and the average size of their oligomers with the number of cooperatively interacting subunits found in the present study. In addition, this model is not in agreement with the structure of the growing ends of microtubules found in the present and previous studies [38]. A similar model derived from an electron microscope analysis of the polymerization of chromatographically purified 6S and 36S depolymerization products has been proposed by

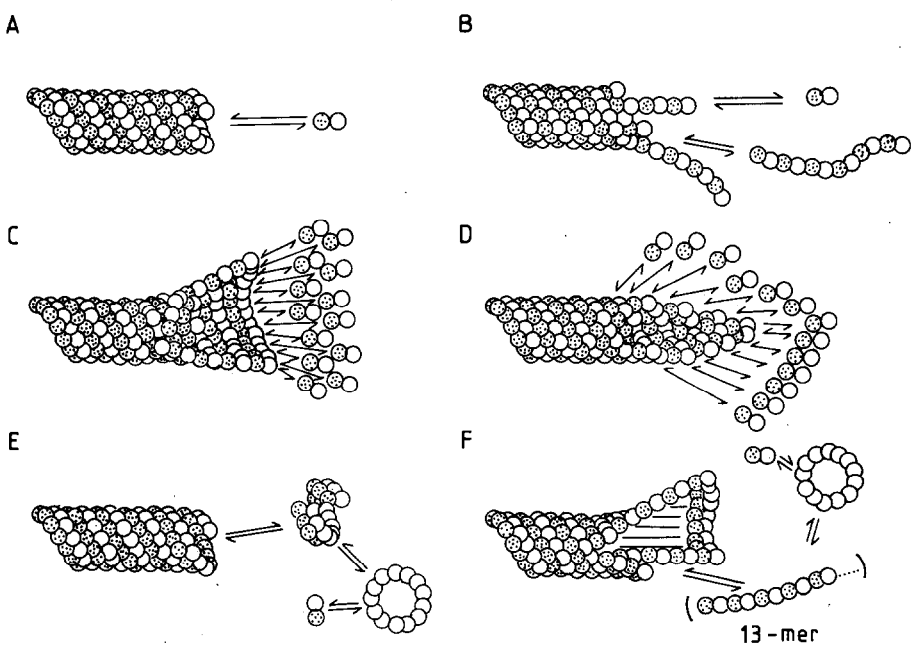

Fig. 5. A, Non-cooperative model of Johnson and Borisy [44] and Gaskin et al. [2]; B, non-cooperative model of Kirschner and Williams [35] and Erickson [34]; C, D, E and F, some possible cooperative models.

Erickson [34]. However, an intergral part of both these models is that in order for elongation to occur, the rings or protofilaments must be continuously generated during the course of polymerization. This requirement seems to be in contradiction with the fact that the rings almost disappear during the initial stages of polymerization [16] and with the finding that chromatographically purified 6S subunits do not form rings or protofilaments [34]. The models presented in Fig. 5C–E depict several ways in which microtubule elongation could occur cooperatively. We feel that the models which are most compatible with the calorimetric and electron microscopic data are the models represented in Fig. 5C and 5D. Both of these are essentially modifications of a model proposed by Bryan [38] whereby elongation occurs by the cooperative addition of 6S dimers to flattened sheets of protofilaments found at the end(s) of growing microtubules, followed by the sealing of the two edges of the sheet to form an intact cylinder. The primary difference between the two models is that in Fig. 5D, when a 6S dimer is added, it is immediately stabilized in both the lateral and longitudinal bonding domains of the protofilament as suggested by Erickson [34]; whereas in Fig. 5C, dimer addition is first stabilized by longitudinal bonds and secondarily by lateral bonds between protofilaments. In Fig. 5D the dimers are stabilized by both longitudinal and lateral bonding due to the staggered array of the ends of the protofilaments. These two models are consistent with the relationship between protein concentration on the one hand and both the degree of cooperation and the appearance of the two ends of growing microtubules on the other. In addition, they provide a functional interpretation for the occurrence of C-shaped microtubules found in thin sections of mitotic apparatus [45–47].

The model in Fig. 5E is a modification of that in Fig. 5A and the model in Fig. 5F is a variation of the model in Fig. 5B. Both are also consistent with the proposed number of interacting dimers. In Fig. 5F, growth is thought to occur by the addition of an "open" 30S subunit, i.e. a protofilament segment composed of dimers bonded in the longitudinal domain, to the flattened sheet of a growing microtubule. The cooperation would arise by the "zipping up" of adjacent protofilaments i.e. formation of lateral bonds between the added and existing protofilament segments. In Fig. 5E rings are in equilibrium with spirals which add 13 dimers in a structural unit; the ring-shaped 30S unit is opened up comparable to the washer conformation in the case of Tobacco mosaic virus [4] and added as a whole to a growing end. Both models are limited by the fact that they include the 30S subunit as an intermediate which is permanently generated from 6S dimers during the course of polymerization. Again, the requirement seems to be at odds with the data concerning the disappearance of the 30S component during the initial stages of microtubule polymerization [16] and the data regarding 6S $\rightleftharpoons$ 30S interconversion [33].

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