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Ionic and Nucleotide Requirements for Microtubule Polymerization in Vitro†

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ABSTRACT: The ionic and nucleotide requirements for the in vitro polymerization of microtubules from purified brain tubulin have been characterized by viscometry. Protein was purified by successive cycles of a temperature dependent assembly–disassembly scheme. Maximal polymerization occurred at a concentration of 0.1 *M* Pipes (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)); increasing ionic strength by addition of NaCl to samples prepared in lower buffer concentrations did not result in an equivalent level of polymerization. Both Na^+ and K^+ inhibited microtubule formation at levels greater than 240 m*M*, with maximal assembly occurring at physiological concentrations of 150 m*M*. Maximal extent of assembly occurred at pH 6.8 and optimal rate at pH 6.6. Inhibition of polymerization was half-maximal at added calcium concentrations of 1.0 m*M* and magnesium concentrations of 10.0 m*M*. EGTA (ethylene glycol bis(β-aminoethyl ether)tetraacetic acid), which chelates Ca^{2+}, had no effect on polymerization over a concentration range of 0.01–10.0 m*M*. In contrast, EDTA (ethylenediaminetetraacetic acid), which chelates both Mg^{2+} and Ca^{2+}, inhibited assembly half-maximally at 0.25 m*M* and totally at 2.0 m*M*. As determined from experiments using Mg^{2+}–EDTA buffers, magnesium was required for polymerization. Magnesium promoted the maximal extent of assembly at substoichiometric levels relative to tubulin, but was maximal for both rate and extent at stoichiometric concentrations. Elemental analyses indicated that approximately 1 mol of magnesium was tightly bound/mol of tubulin dimer. Viscosity development was dependent upon hydrolyzable nucleoside triphosphate, and stoichiometric levels of GTP were sufficient for maximal polymerization. The effect of magnesium in increasing the rate of GTP-dependent polymerization suggests that a Mg^{2+}–GTP complex is the substrate required for a step in assembly.

Microtubules are abundant in eukaryotic cells and many of the functions with which they are associated, such as cellular shape changes and mitosis, are dependent upon the ordered assembly and disassembly of these structures. In order to analyze these processes, attempts have been made to identify the factors controlling the polymerization of microtubules. In vivo studies on the mitotic apparatus (Inoué and Sato, 1967) and other cellular microtubules (see Tilney (1971) and Margulis (1973) for reviews) indicated that the polymerization reaction was endothermic and sensitive to colchicine. However, the recent development of an in vitro system for the formation of microtubules has facilitated more detailed analyses of the molecular mechanisms governing assembly. The polymerization of microtubules in brain extracts was found to be both temperature and colchicine sensitive (Weisenberg, 1972; Borisy and Olmsted, 1972), and to occur under physiological conditions of neutral pH and moderate ionic strength and be maximal in the presence of GTP (Olmsted and Borisy, 1973). In addition, from centrifugation studies, it was inferred that the formation of tubules was dependent upon a specific particulate structure which might be involved in the initiation of tubule growth (Borisy and Olmsted, 1972; Olmsted et al., 1974). In order to define how solution parameters might affect the overall polymerization process and to attempt to determine the dependence of polymerization on intermediate structures, characterization of purified preparations of tubulin was undertaken. This paper describes the ionic and nucleotide requirements for polymerization, and attempts to identify the mechanisms by which these factors might be involved in the cellular regulation of microtubule assembly.

Experimental Procedure

Preparation of Purified Microtubule Protein. Microtubule protein was purified from porcine brain tissue using two cycles of a reversible, temperature dependent assembly scheme described in detail elsewhere (Borisy et al., 1974, 1975). Microtubules polymerized in the second cycle of purification were pelleted by centrifugation (39,000*g*, 30 min) at 37° for subsequent storage and will be referred to as an H_2P fraction. The pelleted protein was frozen in liquid nitrogen and kept at −80° for periods of up to 1 month with no loss of activity. For the majority of experiments, protein was obtained by resuspension of the frozen pellets in P buffer[1] (0.1 *M* Pipes adjusted to pH 6.9 at 23° with 5 *N*

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[1] Abbreviations used are: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)tetraacetic acid; P buffer, 0.1 *M* Pipes adjusted to pH 6.9 at 23°; EDTA, ethylenediaminetetraacetic acid; H_2P, C_2S, purification fractions (pellet at 37°, 0° supernatant) obtained after two cycles of assembly–disassembly; GMPPCP, β,γ-methyleneguanosine triphosphate; GMPPNP, 5′-guanylyl imidodiphosphate; AMPPCP, β,γ-methyleneadenosine triphosphate; AMPPNP, 5′-adenylyl imidodiphosphate.

NaOH) containing 1.0 or 2.5 m*M* GTP. Following depolymerization for 30 min at 0°, the material was centrifuged at 39,000*g* for 30 min at 0°. The resultant supernatant will be referred to as a C_2S fraction and was used for subsequent polymerization studies, usually at a protein concentration of 2–5 mg/ml.

Column Chromatography. For some experiments, protein was chromatographed to remove unbound and/or exchangeable nucleotide and ions. Pelleted protein (H_2P) was resuspended in P buffer to a concentration of 10 mg/ml and the resuspension (5.0 ml) passed through a 1.5 × 12.0 cm Sephadex G-25 column (bed volume of 20 ml) equilibrated with P buffer. Constant volume fractions (1.0 ml) were collected at a flow rate of 1 ml/min and separation of protein from nucleotides assayed by monitoring the absorbancy of the fractions at 280 and 260 nm. Fractions from the void volume were pooled and centrifuged at 39,000*g* for 30 min at 0° to obtain the experimental material. For some experiments, columns were equilibrated in P buffer containing 1 m*M* EDTA.

Elemental Analyses. Samples were prepared by molecular sieve chromatography as described in the text and were analyzed for magnesium by atomic absorption spectrophotometry using a wet ashing preparative procedure. Analyses were carried out by the Elemental Chemistry Laboratory of the WARF Institute, Madison, Wis.

Other Procedures. Viscometric determinations of polymerization and electron microscopic preparation of samples were performed as described previously (Olmsted and Borisy, 1973). Polymerization data are expressed as the maximal value of specific viscosity attained in each sample; instances where the maximal value and final plateau value were different are indicated in the text. Protein determinations were made according to the method of Lowry et al. (1951) using bovine serum albumin as a protein standard.

Results

General Properties of Polymerization of Purified Microtubule Protein. The microtubule protein used in these experiments was purified by a temperature-dependent assembly–disassembly scheme which selected for tubulin on the basis of competence for polymerization. The resultant purified material (C_2S) was composed principally of two components: 80% was tubulin and 15–20% was high molecular weight material which copurified in constant stoichiometry with the tubulin (Borisy et al., 1975). Several lines of evidence have indicated that microtubule assembly was correlated with the presence of the high molecular weight component, and that this material was bound to the polymer (Murphy and Borisy, 1974, in press). Therefore, the ionic and nucleotide requirements for assembly were examined without further fractionation of this material.

In contrast to the porcine brain extracts characterized previously (Olmsted and Borisy, 1973), purified preparations were found to be very stable and little decrease (<10%) in the extent of polymerization occurred even several hours after the resuspension and centrifugation of the purified protein. In the presence of GTP and at protein concentrations of 3–6 mg/ml, the half-time of decay of polymerization for C_2S preparations was 19 hr as compared to 3.5 hr for extracts at the equivalent tubulin concentrations. However, for the individual experiments described in this paper, viscosity measurements were performed simultaneously using aliquots of the same protein preparation.

The overall kinetics of polymerization were similar to those observed for microtubule formation in extracts under the optimal conditions. Upon shifting material from 0 to 37°, there was a rapid increase in viscosity to a maximum value ($t_{1/2}$ of 2–3 min) which was maintained over the 30–40-min time course of the experiment (plateau value). However, depending on the conditions, the rate of increase to the plateau level and the stability of this level varied; instances where the kinetics deviated from this pattern are described. As with the previous determinations on extracts, viscosity development in purified material corresponded to an increase in both the number and length of tubules. Other filamentous aggregates which might have contributed to the viscosity were not observed. In addition, no viscosity increase occurred under conditions where microtubule polymerization was inhibited (colchicine, 0°). Above a critical concentration, viscosity increase was proportional to the protein concentration. As discussed subsequently, the assay of polymerization by quantitative sedimentation corresponded closely to the viscosity assay and therefore the level of viscosity development was taken as an indication of the amount of polymer formed.

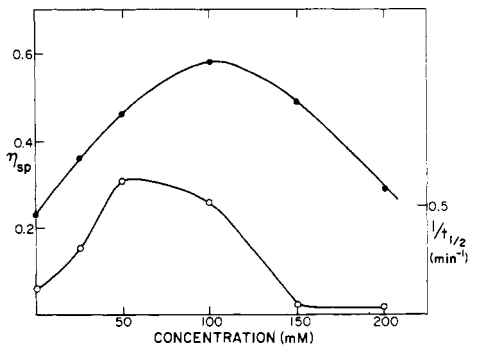

FIGURE 1: Dependence of polymerization on Pipes concentration. Purified protein (H_2P) was resuspended in 10 m*M* Pipes containing 1 m*M* EGTA and 1 m*M* GTP and centrifuged to obtain a C_2S preparation. Aliquots (0.8 ml) were made to various Pipes concentrations by the addition of up to 0.2 ml of a stock solution of 1.0 *M* Pipes containing 1 m*M* EGTA and 1 m*M* GTP. The concentration represents the total added value of Pipes; actual total concentrations are estimated to be approximately 8 m*M* greater (see text). η_{sp} (●) represents the plateau value; $t_{1/2}$ (○) represents the time required to attain half the maximal viscosity level at each Pipes concentration. Protein concentration, 2.6 mg/ml.

Buffer Conditions. The ionic strength conditions for maximal polymerization were investigated by determining the effect of buffer concentration and the addition of other ions on the assembly process. To determine the Pipes concentration optimal for microtubule formation, protein was prepared either by (1) resuspending individual pellets (H_2P) with various concentrations of Pipes containing 1 m*M* EGTA and 1 m*M* GTP and sedimenting to obtain a C_2S preparation or (2) resuspending an H_2P fraction in 10 m*M* Pipes containing 1 m*M* EGTA and 1 m*M* GTP, preparing a C_2S fraction, and then increasing the Pipes concentration by addition of a concentrated stock solution. Results from either type of preparation were similar, and data from an experiment using the latter type of material are shown in Figure 1. In the absence of any added Pipes, a low level of polymerization was observed (initial viscosity level of 0.1). However, since pellets were resuspended in 10 m*M* Pipes and aliquots for polymerization were diluted by 20% with stock solutions, the residual concentration of Pipes in the samples was estimated to be approximately 8 m*M*. With increasing concentrations of added Pipes up to 100

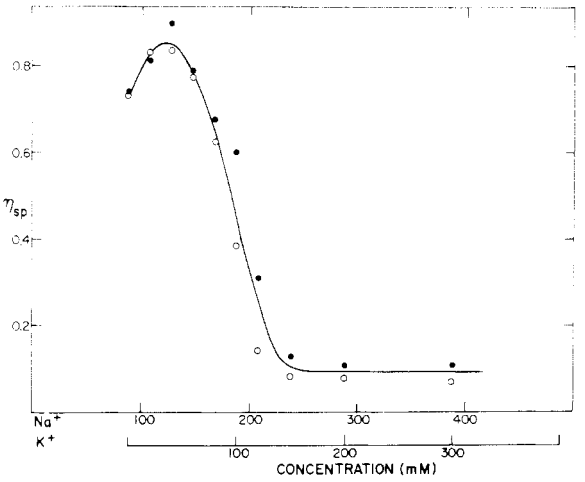

FIGURE 2: Effect of monovalent ions on polymerization. Purified protein (H_2P) was resuspended in 50 m*M* Pipes containing 1 m*M* EGTA and 1 m*M* GTP and centrifuged to obtain a C_2S preparation. Aliquots (0.9 ml) were made to various concentrations with stock solutions of either NaCl (●) or KCl (○). η_{sp} represents the maximum plateau value in each sample. Na^+ concentration represents the total sodium concentration in each aliquot calculated from the amount of added NaCl and the Na^+ content of the Pipes buffer (see text). K^+ concentration represents the total added KCl concentration: this scale also corresponds to the total added NaCl concentration. Protein concentration, 4.5 mg/ml.

m*M*, the extent of polymerization increased; at higher concentrations, viscosity development declined. Although the extent of polymerization was almost symmetrical on either side of the optimum at 100 m*M* added Pipes, there was a marked difference in the rate of polymerization at the lower and higher concentrations. As shown in Figure 1, the time required to attain half the maximal viscosity level in each sample was approximately equivalent for added Pipes concentrations of 50 and 100 m*M*. At higher concentrations, the rate of polymerization was decreased. The final pH of each protein solution (measured at 23°) was determined to be between 6.84 and 6.93 and, as described subsequently, this small variation in pH would not account for the marked difference in rate. On the basis of both extent and rate of polymerization, protein was routinely prepared in 100 m*M* Pipes for optimal assembly.

The effect of increasing ionic strength by the addition of salt rather than Pipes was investigated using procedures similar to those employed for examining the optimum buffer concentration. Protein was prepared in 10 m*M* Pipes or water titrated to pH 6.9 containing 1 m*M* EGTA and 1 m*M* GTP; aliquots of NaCl or KCl were then added to obtain a range of concentrations from 0.05 to 0.2 *M*. As a control, a sample was prepared in P buffer containing 1 m*M* EGTA and 1 m*M* GTP. At added concentrations of 0.05–0.1 *M* NaCl or KCl, some increase in viscosity was observed; however, stimulation was low and varied from 19 to 55% of the value for the control samples. At higher added concentrations of NaCl or KCl (>0.1 *M*), no polymerization was obtained (see subsequent results). Although these data indicated that increasing salt concentration could result in some tubule formation, in no instance was the extent of polymerization similar to that for samples prepared using the standard buffer containing 0.1 *M* Pipes.

Monovalent Cations. The effect of monovalent cations on polymerization was explored further under conditions optimal for assembly. In extract preparations it had been observed that added Na^+ or K^+ suppressed polymerization at all concentrations tested, with total inhibition occurring at added concentrations greater than 150 m*M* (Olmsted and Borisy, 1973). However, because of the additional contribution of these ions from the cellular fluids in the extracts, the total ion concentration in these experiments was undoubtedly higher than the added values. Therefore, experiments were carried out to define further the effect of these ions on assembly in purified material. In the presence of 0.1 *M* Pipes, addition of Na^+ or K^+ ions caused only inhibition of polymerization. Since Pipes buffer is a sulfonic acid adjusted to neutrality with NaOH, it was possible that there was sufficient Na^+ in the buffer solution to mask any stimulation by the monovalent cations. Therefore, samples were prepared in a lower concentration of Pipes (50 m*M* Pipes containing 1 m*M* EGTA and 1 m*M* GTP) such that stimulatory effects of the ions might be detected. As shown previously (Figure 1), this Pipes concentration was sufficient for significant levels of polymerization. From the sodium content of the Pipes (supplied as a lot analysis by the Analytical Chemistry Department of Calbiochem, San Diego, Calif.) and measurement of the amount of NaOH required to titrate the buffer to pH 6.9 at 23°, the total Na^+ content of 50 m*M* Pipes was calculated to be 88 m*M*. The results of an experiment in which samples were made to various concentrations with either NaCl or KCl are shown in Figure 2. At added concentrations of up to 50 m*M* monovalent ion (equivalent to a total concentration of 140 m*M*), a small amount of stimulation of polymerization was observed whereas above this value, the maximal viscosity level declined. Polymerization was half-maximal at total added concentrations of 90 m*M* (total concentration of 180 m*M*), and complete inhibition occurred at added concentrations of greater than 150 m*M* (total concentration of 240 m*M*). Both Na^+ and K^+ produced identical patterns, indicating that stimulation and inhibition were not dependent upon the species but on the total concentration of monovalent ion present. Maximal viscosity development was obtained at a total concentration of Na^+ equivalent to that present in 100 m*M* Pipes (pH 6.9). Therefore, as had been observed for extract preparations, any added ion above this value would inhibit assembly. In addition, optimal polymerization occurred at total Na^+ or K^+ concentrations equal to those estimated for the intracellular monovalent ion concentration in mammalian cells.

pH. The effect of pH on microtubule formation in purified preparations was investigated to determine the optimum for both the rate and extent of assembly. To circumvent the possibility that titration of buffers to alkaline values with NaOH might increase the total Na^+ content to inhibitory levels, protein samples were prepared in 50 m*M* Pipes. Individual pellets (H_2P) were resuspended in buffers (50 m*M* Pipes containing 1 m*M* EGTA and 1 m*M* GTP) which had been adjusted to a range of pH values; C_2S preparations were obtained, the protein concentration of each aliquot was adjusted to an equivalent value, and polymerization was assayed. Results from such an experiment are shown in Figure 3 where the pH represents the final value for the protein solution at 23° (see figure legend). The extent of polymerization was maximal at pH 6.7–6.8 with viscosity development declining symmetrically on either side of this pH value; electron microscopic observations demonstrated that samples at all pH values contained microtubules and few other aggregates. Kinetic data indicated that all samples attained stable plateau levels; however, the rate of viscosity development was maximum at a slightly more acidic pH (6.6) than the optimum for the extent of assem-

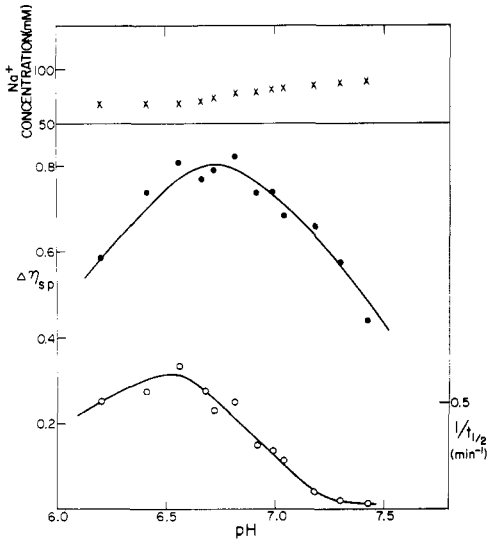

FIGURE 3: Effect of pH on the extent and rate of polymerization. Purified protein (H_2P) was resuspended in individual buffers of 50 mM Pipes, 1 mM EGTA, and 1 mM GTP which had been adjusted to the following pH values at 23°: 5.92, 6.11, 6.37, 6.55, 6.71, 6.83, 6.91, 7.11, 7.19, 7.37, 7.53, 7.80. The samples were centrifuged to obtain C_2S preparations and the corresponding values at 23° for the final samples were respectively: 6.20, 6.41, 6.56, 6.67, 6.73, 6.81, 6.92, 6.99, 7.04, 7.18, 7.30, 7.43 (abscissa). The total sodium concentration (X) in each aliquot was calculated from the Na^+ content of the buffer and the total volume of 5 N NaOH required for titration. η_{sp} (●) represents the maximal viscosity level; $t_{1/2}$ (○) is the time required to attain the half-maximal viscosity level for each sample. Protein concentration, 4.4 mg/ml.

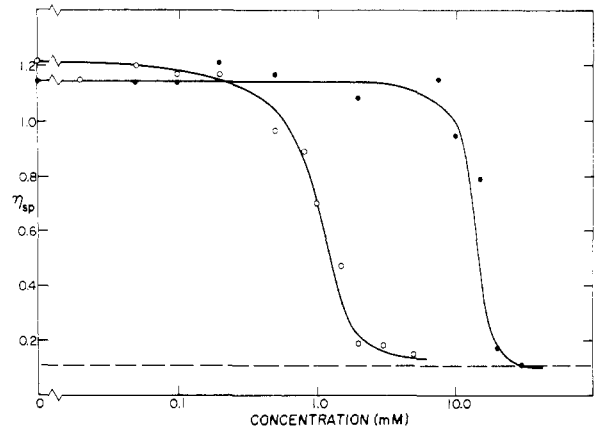

FIGURE 4: Effect of divalent cations on polymerization. Purified protein was prepared in P buffer containing 2.5 mM GTP. Immediately before incubation, aliquots were made to various concentrations with either $CaCl_2$ (○) or $MgCl_2$ (●). η_{sp} represents the plateau viscosity level; the dashed line indicates initial viscosity level. Protein concentration, 5.9 mg/ml.

bly. These observations are in agreement with the data obtained previously for the pH dependence of assembly in extracts (Olmsted and Borisy, 1973). The total Na^+ concentration of the experimental samples varied between 66 and 88 mM (upper panel, Figure 3). Since the previous findings on the dependency of assembly on monovalent cation concentration indicated that this range of values would have little effect on the extent of viscosity development, it was concluded that these data reflected the intrinsic effect of pH upon the assembly process.

Divalent Cations. Previous studies showed that calcium inhibited microtubule polymerization in vitro; however, there was a discrepancy concerning the levels required for inhibition. Weisenberg (1972) calculated that 6 μM calcium was sufficient for complete inhibition. In contrast, we found that millimolar concentrations of calcium were required to block assembly in extracts (Olmsted and Borisy, 1973). Therefore, a more detailed analysis of the possible role of divalent cations in the polymerization reaction was undertaken using purified material.

Inhibition by Calcium and Magnesium. Experiments were initially carried out to determine the effect of Ca^{2+} and Mg^{2+} on the extent of polymerization. Protein was isolated in the absence of EGTA, the purified material resuspended in P buffer containing 2.5 mM GTP and incubation carried out in the presence of varying concentrations of ions. As shown in Figure 4, added concentrations of less than 0.1 mM $CaCl_2$ had little effect on polymerization. The extent of polymerization was half-maximal at 1.0 mM added Ca^{2+}, with total inhibition occurring at concentrations greater than 2.0 mM. Millimolar levels of calcium added to polymerized samples also caused a rapid decline in viscosity and a corresponding depolymerization of tubules. The kinetics of viscosity development showed that there was a uniform depression in the rate of polymerization at the inhibitory concentrations of calcium; however, no effect on rate was observed at the noninhibitory levels. Based on the protein concentration of the samples and the tubulin content of approximately 80%, it was estimated that total inhibition occurred in the presence of a 50-fold molar excess of calcium to tubulin dimer (mol wt 110,000).

The effect of magnesium on microtubule assembly was also investigated. As shown in Figure 4, added magnesium had little effect on the extent of polymerization at concentrations less than 10 mM, inhibited half-maximally at 13 mM, and was totally inhibitory at concentrations greater than 30 mM. However, unlike Ca^{2+}, Mg^{2+} had a marked effect on the rate of polymerization in the noninhibited samples (see Figure 7 and subsequent results). Concentrations at which total inhibition was observed were calculated to represent approximately a 700-fold molar excess of the ion relative to the tubulin dimer.

The affinity constant for Mg^{2+}-nucleoside triphosphate binding is approximately 2.3 times greater than for Ca^{2+}-nucleoside triphosphate interactions ($K = 3.56 \times 10^3\ M^{-1}$ for Ca^{2+}, $8.6 \times 10^3\ M^{-1}$ for Mg^{2+} at pH 6.8, 37°, 165 mM Na^+).[2] Therefore, if the sequestration of the nucleotide by the divalent cations was the basis for inhibition by either species, magnesium would be expected to be inhibitory at lower concentrations than calcium. Since inhibitory Mg^{2+} concentrations are tenfold greater than those for calcium, these data suggest that inhibition of microtubule formation by these ions occurs by another mechanism (see Discussion).

To determine whether Mg^{2+} and Ca^{2+} might have synergistic effects on the inhibition of polymerization, experiments were designed to investigate whether magnesium concentration altered the level of calcium required to block tubule formation. As shown in Table I, at magnesium concentrations which were sufficient for maximal polymerization or were slightly inhibitory (10 mM) the concentrations

[2] Affinity constants for Ca^{2+}-nucleoside triphosphate and Mg^{2+}-nucleoside triphosphate interactions were derived using data from O'Sullivan and Perrin (1964). Since the affinity constants are dependent on Na^+ content, temperature, and pH, the values were calculated for the conditions under which the experimental material was used (37°, 0.165 M Na^+, pH 6.8, for the final protein solution).

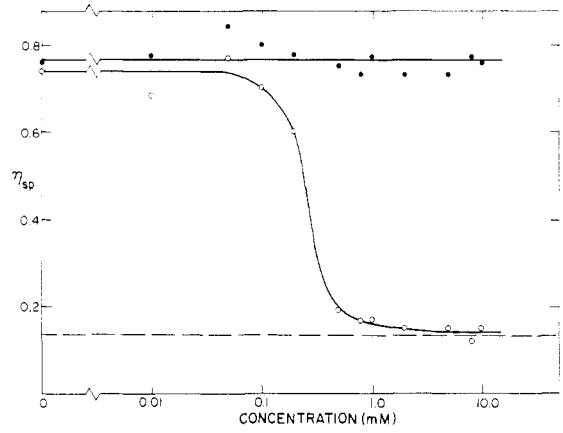

FIGURE 5: Effect of chelators on polymerization. Purified protein was prepared in P buffer containing 1.0 m*M* GTP. Immediately before incubation, aliquots were made to various concentrations with either EGTA (●) or EDTA (○). η_{sp} represents the plateau viscosity; the dashed line indicates initial viscosity level. Protein concentration, 3.6 mg/ml.

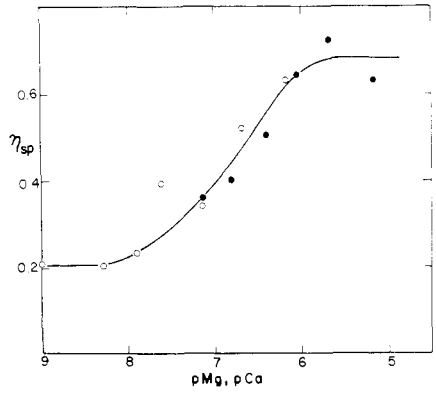

FIGURE 6: Effect of Mg^{2+} and Ca^{2+} on polymerization in the presence of EDTA. Purified protein was prepared in P buffer containing 1 m*M* EDTA and 1 m*M* GTP. Samples (0.9 ml) were made to various concentrations with either $MgCl_2$ (●) or $CaCl_2$ (○); the final concentration of EDTA and GTP in the samples was 0.9 m*M*. pMg and pCa are the calculated free concentrations of the respective ions, taking into account chelation by both EDTA and GTP (see text). η_{sp} represents the viscosity level after 30-min incubation. The viscosity value for an unpolymerized sample was 0.1. Protein concentration, 3.3 mg/ml.

of calcium required for half-maximal inhibition of assembly were between 10^{-3} and 10^{-4} *M*. At magnesium concentrations (20 m*M*) where significant inhibition was observed, inhibition by calcium occurred at lower concentrations (3×10^{-5} *M*). These data indicated that magnesium concentration did affect the levels of calcium required for inhibition, although only at high concentrations where Mg^{2+} alone was also inhibitory.

Chelators. The preceding experiments indicated that the extent of polymerization was equivalent for samples incubated in the absence of added divalent cations or with noninhibitory concentrations of cations. These data suggested either that these ions were not required for polymerization, or that sufficient magnesium or calcium was present in solution and/or bound to the protein to promote assembly. To distinguish between these possibilities, further experiments using chelating compounds were carried out to define the divalent cation requirement for polymerization. Protein was prepared in the absence of any chelator and then polymerized in the presence of various concentrations of EGTA or EDTA. EGTA chelates calcium strongly but magnesium

Table I: Effect of Mg^{2+} Concentration on Inhibition of Polymerization by Ca^{2+}.[a]

$MgCl_2$ (m*M*)	Extent of Polymerization[b] (%)	$CaCl_2$[c] (m*M*)
0	100	0.85
0.1	99	0.72
5.0	109	0.52
10.0	67	0.38
20.0	29	0.03

[a] Protein was prepared in P buffer containing 1.0 m*M* GTP and made to various Mg^{2+} and Ca^{2+} concentrations immediately before polymerization. Protein concentration, 3.6 mg/ml. [b] The change in viscosity (η_{sp} plateau − η_{sp} initial) for a sample with no added magnesium was designated as 100% polymerization; the remaining figures were obtained relative to this value. [c] Calcium concentration curves were obtained at each magnesium concentration. Value represents the calcium concentration at which half-maximal inhibition of polymerization was observed relative to a sample with no added calcium at the given magnesium concentration.

only weakly (at pH 6.8, $K = 3.14\ M^{-1}$ for Mg^{2+}, $K = 1.94 \times 10^6\ M^{-1}$ for Ca^{2+}) whereas EDTA complexes both Ca^{2+} and Mg^{2+} strongly (at pH 6.8, $K = 1.12 \times 10^7\ M^{-1}$ for Ca^{2+}, $1.42 \times 10^5\ M^{-1}$ for Mg^{2+}).[3] As shown in Figure 5, EGTA had no effect on the extent of microtubule formation and the kinetics of polymerization at all concentrations were similar to those for samples to which no chelator had been added. In contrast, addition of EDTA had a marked effect on polymerization. Half-maximal inhibition of assembly occurred at concentrations of 0.25 m*M* and total inhibition at concentrations greater than 2.0 m*M;* at all inhibitory concentrations, there was a marked decrease in the rate of assembly, although all samples attained stable plateau values. Since EDTA chelates Mg^{2+} and Ca^{2+} strongly whereas EGTA complexes only Ca^{2+} effectively, these data indicated that magnesium was required for assembly.

To obtain a more accurate estimate of the free divalent ion concentration required for assembly, polymerization was examined in the presence of metal–EDTA buffers. Protein was incubated in the presence of various concentrations of calcium or magnesium and with 0.9 m*M* EDTA. Taking into account the amount of added ion complexed with both EDTA and GTP,[4] the extent of polymerization as a function of free ion concentration was determined and plotted as the specific viscosity developed after 30-min incubation at 37° (Figure 6). After this time, polymerization had attained stable plateau values for samples containing above 0.5 μM free magnesium or calcium. For samples containing less than this amount of free divalent cation, kinetic curves indicated that the extent of polymerization was still gradually increasing although the maximum in rate had been achieved. In the absence of any added ion, there was only a slight increase in viscosity above the initial level of 0.1 and an equivalent level of polymerization was seen for samples which contained below 10^{-8} *M* free calcium or magnesium. At free concentrations of either ion between approximately 10^{-8} and 10^{-7} *M*, there was a gradual increase in the specific viscosity over the basal value. Maximal stimulation oc-

[3] Data for affinity constants for chelators were obtained from Caldwell (1970) and calculated for pH 6.8.

[4] The free Mg^{2+} or Ca^{2+} concentration was determined by successive approximation using the affinity constants for both the nucleotide–ion and chelator–ion complexes.

curred at free concentrations of either calcium or magnesium greater than 0.8 μM (0.1 mM added Mg^{2+}; 0.8 mM added Ca^{2+}) and the extent of polymerization was equivalent to that for a sample to which no EDTA or ion was added. At higher concentrations (data not shown), inhibition was observed when the calculated free magnesium concentration exceeded 8.0 mM and the calcium concentration 0.5 mM; these data were therefore consistent with the results on inhibition of polymerization obtained previously (Figure 4).

Since the addition of magnesium overcame the inhibitory effect of EDTA, the results of these experiments support the conclusion that inhibition of polymerization by EDTA was due to the sequestration of ions, and not due to an anomalous effect of the chelator on the protein. Additional information consistent with this interpretation was obtained from analyses of the kinetics of assembly in the presence and absence of chelator and are discussed subsequently. In addition, these data on the effect of chelators on polymerization demonstrated that magnesium was required for polymerization, although either magnesium or calcium at micromolar levels could stimulate assembly following inhibition by EDTA.

Elemental Analyses. Since the chelator experiments had demonstrated that magnesium was required for microtubule assembly, analyses were carried out to determine whether this ion was bound to the tubulin subunit. To remove ion and/or nucleotide in the preparations which might not be bound to the protein, pelleted protein (H_2P) was resuspended in P buffer and chromatographed on a column of G-25 Sephadex equilibrated in the same buffer. The voided fractions were pooled, centrifuged to obtain a C_2S fraction, and analyzed for ion content by atomic absorption spectrophotometry. Analyses of the buffer alone demonstrated that magnesium content was between 0.5 and 1.0 μM. The protein sample (5.0 mg/ml) had an estimated molar concentration of tubulin of 36 μM and a magnesium content of 1.0 μg/ml (41.6 μM). Assuming that the magnesium was complexed only with tubulin, these data indicated that a maximum of 1.15 mol of magnesium was bound/tubulin dimer. Analyses carried out on protein prepared and chromatographed in P buffer containing 1 mM EDTA gave similar molar ratios and indicated that the magnesium was probably tightly complexed (see Discussion).

Although technical difficulties were encountered in carrying out similar analyses for calcium, preliminary results indicated that the protein contained approximately a fivefold lower molar concentration of calcium than magnesium, and negligible amounts were present in the buffer.

Dependence of Rate on Magnesium. Since the chelator data had indicated that Mg^{2+} stimulated polymerization at extremely low levels, further analyses were made on the effect of magnesium concentration on the kinetics of assembly. In the absence of any chelator (Figure 7), the extent of polymerization was equivalent over a wide range of magnesium concentrations. However, there was a marked effect of the ion on the rate of viscosity development, with maximal stimulation at added concentrations of approximately 0.2 mM. Similar analyses were performed at 24° in order to obtain more complete kinetic data. Under these conditions, the time required to reach maximum viscosity levels was approximately tenfold greater than at 37° (18 min at 24° as compared with less than 2 min at 37° at 0.1 mM Mg^{2+}). The optimal rate of polymerization was determined to occur at added magnesium concentrations of 0.1–1.0 mM and

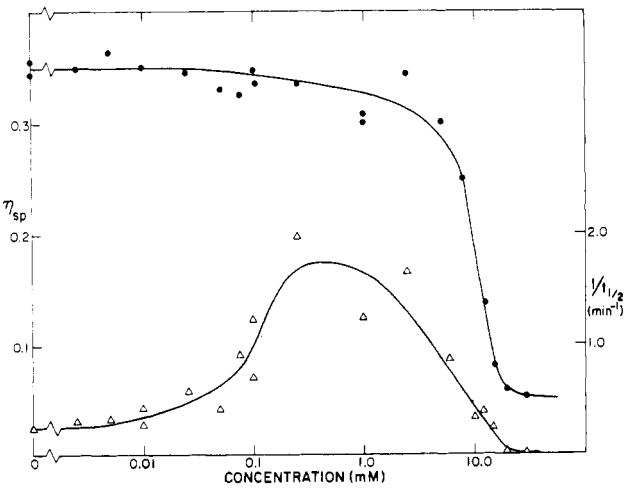

FIGURE 7: Effect of Mg^{2+} on the extent and rate of polymerization. Purified protein was prepared in P buffer containing 2.5 mM GTP. Immediately before incubation, aliquots were made to various concentrations with $MgCl_2$. η_{sp} (●) represents the maximum viscosity level; $t_{1/2}$ (△) corresponds to the time required to attain the half-maximal viscosity value in each sample. Protein concentration, 2.1 mg/ml.

therefore confirmed the results obtained at 37°. In the presence of 1 mM EDTA, similar calculated free levels of Mg^{2+} (0.1–1.0 mM) were required for maximal rate of polymerization at 24°. Since the maximum rate of polymerization in the presence or absence of EDTA occurred at equivalent concentrations of free magnesium, these data confirmed that the inhibition of assembly by EDTA was specifically due to the chelation of the magnesium required for polymerization. As calculated from the protein concentrations of the incubated samples in all of these experiments, the maximal rate of polymerization occurred when magnesium was present at equivalent or greater than equimolar levels compared to tubulin. However, since some stimulation of polymerization occurred when free Mg^{2+} concentrations were less than equimolar (Figure 6) we infer that magnesium is involved catalytically in the polymerization reaction (see Discussion).

Nucleotide Dependence of Polymerization. It had been observed previously that microtubule protein isolated from many sources had associated guanine nucleotides (Shelanski and Taylor, 1968; Weisenberg et al., 1968; Bryan, 1972) and our observations on extracts (Olmsted and Borisy, 1973) and purified material (Borisy et al., 1974; see below) had demonstrated that nucleoside triphosphate was required for polymerization. In light of the dependence of assembly on the presence of magnesium and the precedent for Mg^{2+}–nucleotide complexes being the reactive species in many nucleotide-dependent reactions, a more detailed analysis of the nucleotide requirements for microtubule polymerization was undertaken.

To investigate the concentration of GTP required for polymerization in purified material, protein was prepared by passage over a G-25 Sephadex column equilibrated in P buffer to remove nucleotide from the exchangeable binding site (see Discussion). The voided fractions were pooled, centrifuged at 0°, and then made to varying GTP concentrations and 0.1 mM Mg^{2+}. As shown in the insert of Figure 8, no polymerization was observed in the absence of added GTP or at added concentrations less than 1 μM. At concentrations between 2 and 10 μM, there was a progressive increase in the extent of polymerization, and at concentrations greater than 50 μM, maximal viscosity levels were ob-

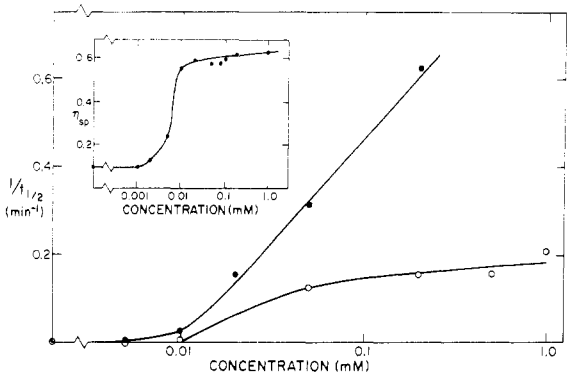

FIGURE 8: Dependence of polymerization on GTP concentration and Mg^{2+}. Protein (H_2P) was resuspended in P buffer and passed over a G-25 Sephadex column equilibrated in P buffer, and the voided fractions were pooled and centrifuged to obtain a C_2S preparation. Aliquots were prepared in the presence (●) or absence (○) of 0.1 m*M* $MgCl_2$, and made to various GTP concentrations (abscissa). $t_{1/2}$ represents the time required to attain half the maximal viscosity level in each sample. Protein concentration, 4.2 mg/ml. Insert: Protein was prepared as above in the presence of 0.1 m*M* $MgCl_2$ and made to varying GTP concentrations. η_{sp} represents the maximal viscosity level attained regardless of stability (see text). Protein concentration, 3.8 mg/ml.

served. However, examination of kinetics indicated that although maximal polymerization was achieved at concentrations as low as 10 μM, stable plateau values were generally not maintained unless GTP was present at concentrations greater than 200 μM; at lower GTP concentrations (<50 μM), there was a relatively larger decline in the viscosity value. Based on the tubulin content and protein concentration of the samples, it was estimated that approximately stoichiometric levels of GTP were sufficient for maximal levels of polymerization, although concentrations in excess of stoichiometric values (e.g., 0.5 or 1.0 m*M*) were routinely used in order to maximize the stability of the plateau.

Similar data on the concentration of GTP required for the maximal extent of polymerization were obtained for identical preparations incubated in the absence of added magnesium. In theory, the chromatography procedure should have removed excess magnesium from the protein preparations, and one might have predicted that such preparations would not polymerize. However, in the absence of chelator, the presence of low levels of magnesium in these preparations could not be excluded. As determined using EDTA buffers, the free magnesium concentrations which were sufficient for maximal polymerization were less than 0.8 μM, and the elemental analyses indicated that the magnesium content of 0.1 *M* Pipes was approximately 0.5–1.0 μM, and added concentrations of GTP up to 1 m*M* contributed a maximum of 0.2 μM. Therefore, sufficient Mg^{2+} remained in these preparations to allow maximal polymerization. However, in comparing the kinetic curves for the GTP concentration dependence of polymerization, a difference in the rate of assembly at various GTP concentrations was observed which was dependent upon the presence of added magnesium. As shown in Figure 8, there was a marked increase in the rate of viscosity development in the presence of magnesium; in contrast, there was only a slight increase in rate at GTP concentrations greater than 50 μM in the absence of added magnesium. Both types of preparations attained the same maximal level of viscosity at equivalent GTP concentrations and these data suggested that the magnesium was limiting for the rate of the reaction. Since maximal stimulation of viscosity development could occur when substoichiometric levels of free magnesium were present (Figures 6 and 8), this effect may be indicative of the formation of a transient Mg^{2+}-GTP complex which is required for the promotion of rapid polymerization (see Discussion).

The specificity of polymerization for both the species of nucleotide and the nature of the phosphate moiety had been examined previously (Borisy et al., 1974). These data had demonstrated that although the extent of polymerization was essentially equivalent in material prepared with ATP, CTP, GTP, or UTP, no viscosity increase was observed in samples incubated with nucleotides which lacked a γ-phosphate group (e.g., GMP, GDP, cGMP, or cAMP) or in which the terminal phosphate group was nonhydrolyzable (GMPPCP). Although these results suggested that nucleotide hydrolysis was required for polymerization, it was possible that the other compounds tested had a lower affinity for binding to the protein and polymerization was therefore not observed. Since the recent data had indicated that low levels of GTP (100 μM) were sufficient for assembly and that magnesium promoted rapid tubule formation, the effect of nonhydrolyzable analogs was reexamined under conditions where the optimum rate of polymerization would be favored. When protein was incubated in the presence of 0.1 m*M* Mg^{2+} and 1.0 m*M* concentrations of GTP or ATP or nonhydrolyzable analogs of these nucleotides (GMPPNP, GMPPCP, AMPPNP, AMPPCP), viscosity developed only in those samples containing the hydrolyzable nucleoside triphosphates. Therefore, these data again suggested that hydrolysis was required for assembly. However, to test whether analogs were actually binding to the protein, samples were prepared which contained various concentrations of both GMPPCP and GTP. At a GTP concentration sufficient for maximal assembly (0.1 m*M*), added concentrations of GMPPCP of 0.5, 1.0, and 2.0 m*M* inhibited the extent of polymerization, respectively, by 22, 69, and 86% compared to the control sample (no added GMPPCP). At lower GTP concentrations where polymerization was suboptimal, greater degrees of inhibition were observed at lower GMPPCP concentrations; in contrast, at higher GTP concentrations (0.5, 1.0 m*M*), GMPPCP at all tested concentrations had little or no effect. These data suggested that GMPPCP could compete with GTP and thereby inhibit polymerization. Therefore, inhibition of assembly in samples prepared with nucleotide analogs is inferred to be due to the lack of a hydrolyzable phosphate group required for polymerization rather than to the inability of the analog to bind to the protein.

Discussion

The experiments described in this report were undertaken to define the ionic requirements for polymerization of purified microtubule protein. The pH and Na^+ and K^+ concentrations found to be optimal for polymerization were analogous to the pH (Waddell and Bates, 1969) and monovalent cation concentration reported to exist in vivo. In addition, as discussed subsequently, the levels of magnesium which promoted the polymerization of brain tubulin were similar to the intracellular concentrations determined to be present in nervous tissue (Veloso et al., 1973). Therefore, the conditions optimum for in vitro polymerization appeared to be physiological with respect to many of the ionic parameters examined. However, although levels of monovalent cations (or total ionic strength) reported by other workers (Haga et

al., 1974; Lee et al., 1974; Kuriyama and Sakai, 1974) to be optimal for assembly were similar to those which we observed, differences existed in the data obtained on the pH dependence of polymerization. Lee et al. (1974) described a pH optimum for rate of 6.0–6.4, with little assembly occurring at pH 7.0 and Haga et al. (1974) observed that there was a decline in the extent of polymerization as pH was raised from 6.2 to 7.3. Since differences exist in the buffers used, the methods of measuring polymerization, and the preparation of tubulin, it is difficult to evaluate the basis for the disparity between these experimental data.

The results on the dependence of viscosity development on buffer concentration demonstrated that there was an optimum Pipes concentration for assembly and that varying ionic strength with added salt did not result in the equivalent level of polymerization. Therefore, in contrast to the in vitro polymerization of flagellin (Wakabayashi et al., 1969) and tobacco mosaic virus protein (Durham, 1972), increasing ionic strength to values greater than 0.15 did not facilitate assembly. However, although a total Na^+ concentration of 240 mM was inhibitory when obtained by addition of NaCl to 50 mM (or 100 mM) Pipes, a similar total Na^+ concentration present in 150 mM Pipes did not prevent tubule formation. Although these data could be interpreted as indicating that the chloride anion had a more inhibitory effect than the Pipes anion, our results and other observations suggest that Pipes may actually stabilize tubulin and thereby favor assembly. As originally observed by Weisenberg (1972), several of the buffers described by Good et al. (1966), including Pipes, promoted microtubule polymerization, whereas no assembly occurred in other commonly used buffers (e.g., phosphate). Most of the "Good" buffers have zwitterionic properties and, therefore, at pH values near their pK value, have strong dipole moments. Since it has been concluded that many polymerization reactions occur with the loss of structured water from the monomeric unit (Caspar, 1966), it could be postulated that the dipolar nature of these buffers is involved in altering the hydration state of the tubulin subunits and thereby promoting polymerization. Zwitterions such as amino acids are abundant in cells, and it will be of interest to determine if such naturally occurring compounds might be significant in the in vivo control of microtubule assembly.

As observed previously by others (Weisenberg, 1972; Haga et al., 1974; Kuriyama and Sakai, 1974; Lee et al., 1974), microtubule formation was dependent on magnesium. Although we found that maximal polymerization could occur in the presence of substoichiometric levels of free magnesium the optimal rate of assembly was observed at concentrations equivalent to or greater than the molar concentration of tubulin. A comparison of the levels required for in vitro assembly of brain tubulin with the free magnesium found in neurons (Veloso et al., 1973) indicates that these concentrations are in the same range (0.1–1.0 mM). Similar results on the concentrations of magnesium required for polymerization were obtained by Haga et al. (1974).

Inhibition of polymerization by Mg^{2+} (>10 mM) and calcium (>1 mM) occurred at concentrations which were significantly greater than the total levels estimated for the free intracellular concentrations of these ions (1 mM for magnesium; 10^{-6} M for calcium). Therefore, in contrast to the findings of others (Weisenberg, 1972; Haga et al., 1974; Rosenfeld and Weisenberg, 1974), our data do not suggest that changes at the micromolar level of calcium might be involved in the cellular regulation of tubule polymerization. Under the conditions of physiological ionic strength and pH used in our preparations, calcium was inhibitory at 10^{-5} M only at concentrations of magnesium which were 20 times greater than that determined for free intracellular concentrations (1 mM). In addition, these magnesium levels were two times greater than those estimated for total intracellular magnesium concentrations (Veloso et al., 1973), and were sufficient to cause significant inhibition (>70%) of polymerization in vitro. Because of these disparities, it is difficult to postulate a mechanism for the role of calcium in the regulation of tubule polymerization in vivo. Furthermore, the chelator data and the atomic absorption analyses indicate that magnesium and not calcium is the ion bound to freshly isolated tubulin. As discussed subsequently, it is apparent that magnesium is required for assembly. Calcium can substitute for magnesium in the polymerization reaction in vitro, but values for the in vivo levels of these ions indicate that assembly of tubules in living cells involves polymerization of a magnesium–tubulin complex.

The basis for inhibition of in vitro assembly by high concentrations of magnesium and calcium has been postulated from data on the effect of these ions on intermediates involved in polymerization. Centrifugation studies had indicated that the formation of tubules in extract preparations was dependent upon a fraction which sedimented at high speed (Borisy and Olmsted, 1972) and it was suggested that a specific disc structure was required for tubule growth. Several lines of evidence have indicated that the disc (or ring) was an oligomer of tubulin in equilibrium with subunits and that disruption of this structure prevented assembly (Borisy et al., 1975). However, neither magnesium nor calcium at inhibitory concentrations caused the dissolution of discs, and it was suggested (Kirschner et al., 1974; Rosenfeld and Weisenberg, 1974) that the ions might stabilize these structures. Since we have observed that discs disappear rapidly upon the formation of tubules (Olmsted et al., 1974), inhibition by calcium or magnesium may be effected by preventing changes in the subunit–disc equilibrium or disc–polymer transition which are requisite for assembly.

Examination of the nucleotide dependence of polymerization in purified preparations demonstrated that hydrolyzable nucleoside triphosphate was required for assembly, and that levels of GTP estimated to be stoichiometric to tubulin were sufficient for maximal polymerization. Although these absolute values could only be approximated on the basis of the protein concentration of the incubated sample, a quantitative sedimentation assay in which the amount of monomer and polymer formed was measured directly corroborated the viscosity results (Johnson and Borisy, 1974). When data from viscosity and sedimentation assays were obtained from samples at two different protein concentrations, maximal polymer formation and viscosity development were determined to occur at GTP concentrations stoichiometric to the tubulin dimer (1 mol of GTP/mol of tubulin dimer incorporated into polymer). These results were in contrast to the viscometric measurement of GTP concentration dependence obtained in extract preparations (Olmsted and Borisy, 1973); under these conditions, millimolar concentrations calculated to be in 50- to 100-fold excess of the tubulin were required. However, since other proteins present in the extracts might hydrolyze GTP, the higher level observed in these experiments probably does not directly reflect the absolute concentration of GTP required for microtubule assembly.

It is pertinent to examine the results on the magnesium and GTP requirements for polymerization in relation to those which have been obtained on the nucleotide-binding sites for tubulin and their possible role in assembly. It was originally observed that the tubulin dimer contained 2 mol of guanine nucleotide, one of which was readily exchangeable and the other of which was tightly bound (Weisenberg et al., 1968); recent studies by Jacobs et al. (1974) have refined the kinetics of these binding reactions. From the data in this report, we postulate that exchangeable and nonexchangeable sites also exist for the binding of magnesium. Under the conditions used to examine both the GTP dependence of polymerization and the amount of magnesium bound in our material, purified preparations were chromatographed on Sephadex columns. In theory, this procedure would remove any nucleotide and/or ions which were present either free in solution or bound at the exchangeable site, and the voided material would contain nucleotide and/or ion which was tightly complexed with the protein. These preparations were found to have magnesium bound in the ratio of 1 mol/mol of tubulin dimer and preliminary analyses of perchloric acid precipitated protein indicated the presence of 0.5–1.8 mol of nucleotide. The persistence of 1 mol of magnesium/dimer on material incubated and chromatographed with 1 m*M* EDTA indicated that 1 mol of magnesium per dimer was essentially nonexchangeable. However, the inhibition of polymerization by EDTA suggested the presence of a second magnesium per dimer which was exchangeable. The second magnesium is postulated because EDTA was demonstrated to inhibit polymerization by sequestration of magnesium, yet to have no effect on the one magnesium found to be tightly bound to the protein. Although it is currently unknown whether magnesium at either site is bound directly to tubulin or with nucleotide, it can be inferred that the magnesium and nucleotide requirements defined for polymerization reflect the binding of these moieties at the exchangeable site(s).

Maximum polymerization required the presence of concentrations of GTP equimolar to tubulin, and maximum rate of polymerization occurred when magnesium was also present at approximately stoichiometric levels relative to GTP and tubulin. These data suggest that a Mg^{2+}–GTP complex may be the substrate involved in assembly. Since hydrolysis of GTP occurs at the exchangeable site during polymerization (Jacobs et al., 1974), the formation and/or binding of the Mg^{2+}–GTP complex at this site could be a rate-determining step in the reaction. Therefore, it can be postulated that in the presence of substoichiometric levels of magnesium, the rate of the reaction is limited by the recycling of the magnesium to form additional substrate, even though an equivalent final extent of polymerization would be achieved relative to samples containing higher concentrations of ion. This interpretation is directly supported by the data obtained on material prepared by chromatography and assayed for polymerization in the presence of varying GTP concentrations (Figure 8). Equivalent maxima but different rates were obtained at comparable GTP values where one set of samples contained substoichiometric levels of residual magnesium (less than 1.5 μM compared to 28 μM tubulin) and the other samples a fourfold excess of magnesium (0.1 m*M*). In addition, similar conclusions can be derived from the observation that maximal extent of polymerization could occur in the presence of micromolar levels of free magnesium (Figure 6). Hence, at low concentrations, magnesium may be considered to act catalytically in the assembly process. A similar phenomenon of magnesium determining the rate of assembly has been observed for actin polymerization (Oosawa and Kasai, 1971). Although Jacobs et al. (1974) indicated that nucleotide at both the exchangeable and nonexchangeable sites was retained in the polymer (as GDP), it is not yet known whether the added magnesium remains bound to tubulin, is complexed with nucleotide remaining at the exchangeable site, or is released upon hydrolysis of the GTP to GDP during polymer formation.

In conclusion, this report has defined some of the ionic conditions required for microtubule assembly from purified tubulin, and these have been found to be similar to intracellular concentrations of ions. Although it is not yet possible to identify specifically whether changes in ionic or nucleotide concentrations might be involved in the cellular regulation of assembly, it is clear that these factors are significant in affecting the rate and extent of polymerization in vitro. Theories on the cellular control of microtubule polymerization based on calcium as a regulatory ion must be considered with caution since the in vitro data demonstrate that both calcium and magnesium ions inhibit or stimulate polymerization depending on solution conditions.

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The Isolation and Characterization of Nuclear Ghosts from Cultured HeLa Cells†

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ABSTRACT: Macromolecular complexes, which appear as ghosts when viewed by phase contrast microscopy, have been isolated from the nuclei of HeLa cells grown in culture. The preparation of these ghosts involves a detergent wash which removes the unit membranes of the nuclear envelope structure but leaves intact both the nuclear pores and the dense structure conferring nuclear margins (possibly the dense lamella). Detergent-washed nuclei are subsequently treated with 0.5 *M* $MgCl_2$ and fractionated on continuous sucrose gradients containing 0.5 *M* $MgCl_2$. The ghosts are recovered as a sharp band at an apparent sucrose density of 47–52% and consist of 72% protein, 10% phospholipid, 14% DNA, and 4% RNA. The release of the majority of intranuclear components is indicated by the large loss of nuclear DNA (95%), RNA (71%), and protein (87%) contrasted to the small loss of phospholipid (27%) during the conversion of detergent washed nuclei to isolated ghosts. Sodium dodecyl sulfate–polyacrylamide gel patterns of the ghost proteins consist of two major bands with approximate molecular weights of 20,000 and 35,000. The isolation of ghosts with a similar density and protein composition from nondetergent-washed nuclei indicates that the ghost is not an artifact induced by the detergent treatment. The absence of cytoplasmic contamination in the preparations of detergent washed nuclei and nuclear ghosts was demonstrated by chemical, enzymatic, and electron microscope studies. We suggest that the isolated ghosts represent a structural macromolecular complex which underlies and is probably attached to the inner nuclear membrane of intact nuclei. The possible additional presence of intranuclear network proteins has not been excluded.

Investigations into the biochemical behavior of the nuclear envelope during the cell cycle, with particular regard to its cyclic dissolution and re-formation in mitosis, would be greatly facilitated by the isolation of well-defined components of the nuclear envelope from cells grown in culture. Until the present, no attempt at characterization of nuclear envelope fractions prepared on a large scale from cultured cells has been reported, even though there have been a number of studies on nuclear envelopes isolated from tissue sources (e.g., Kashnig and Kasper, 1969; Zbarsky et al., 1969; Franke et al., 1970; Agutter, 1972; Berezney et al., 1972; Bornens, 1973; Monneron et al., 1972).

As a result of ultrastructural studies performed on a wide variety of systems, the nuclear envelope has been recognized as having membranous as well as nonmembranous components. These include the inner and outer nuclear membranes, the dense lamella, the heterochromatin layer, and the annuli or pore structures, which occur in varying numbers on the surfaces of nuclei (Kay and Johnston, 1973).

Although many authors have emphasized the membranous nature of their isolated nuclear envelope fractions, recent evidence suggests that the presence in these fractions of nonmembranous nuclear envelope components may need to be considered as well. For example, while our work was in progress, Aaronson and Blobel (1974a) demonstrated that the nuclear pores, which are often taken to be diagnostic for nuclear membrane, seem to be firmly embedded in a layer (dense lamella or heterochromatin layer) normally internal to both inner and outer nuclear membranes. These investigators have shown that the structural integrity of nuclei is maintained in the absence of any membrane. Furthermore, there have been reports of fibrous protein networks which are continuous with the nuclear envelope and extend throughout the nuclear volume (Smetana et al., 1963; Steele and Busch, 1966; Narayan et al., 1967; Berezney and Coffey, 1974). Despite their obvious importance in maintaining the structure of the nuclei, the likely presence of nonmembranous nuclear and nuclear envelope com-

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