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Characterization of Microtubule Assembly in Porcine Brain Extracts by Viscometry†

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ABSTRACT: The assembly of microtubules in extracts of porcine brain tissue has been characterized by viscometry. The optimal conditions for preparing extracts have been defined in terms of the rate and extent of polymerization, and the stability of the maximal level. The reaction is endothermic, and maximal viscosity levels were attained within 15–20 min of incubation at 37°. No polymerization occurred at temperatures less than 15°, and the polymer was unstable at temperatures greater than 40°. The greatest polymerization occurred over a pH range of 6.7–6.9, and no increase in viscosity was observed at a pH of less than 5.8 or greater than 7.6. Guanosine triphosphate (GTP) augmented polymerization, producing a stable viscosity level at concentrations higher than 2.0 mM. However, at GTP concentrations greater than 3.0 mM, the initial rate of assembly was depressed. At a concentration of 2.5 mM, extracts prepared with other nucleoside triphosphates (ATP, CTP, UTP) or guanosine nucleotides (GDP, GMP) polymerized to a lesser degree than those extracts made with GTP. The extent of viscosity development was approximately proportional to the protein concentration of the extract over the range of 4–12 mg/ml; no polymerization occurred at lower protein concentrations. Viscosity development was completely inhibited by the addition of both monovalent and divalent cations at the following concentrations: Na^+ and K^+, 150 mM; Mg^{2+}, 10 mM; Ca^{2+}, 1 mM. Colchicine, which binds specifically to microtubule subunits, totally inhibited polymerization at a concentration of 1 μM. The conditions established in this paper will enable the use of optimum parameters for further investigations on microtubule assembly.

Microtubules are a common feature of eukaryotic cells, and several reviews on the cellular distribution, function, and biochemical composition of these organelles have appeared (Porter, 1966; Tilney, 1971; Olmsted and Borisy, 1973; Wilson and Bryan, 1973). Until recently, however, detailed information on the properties of microtubule assembly and disassembly was limited. Investigations on mitotic spindle formation and dissolution (Inoué and Sato, 1967) as well as other observations on cellular processes (see Tilney (1971) and Margulis (1973) for reviews) indicated that assembly *in vivo* was both endothermic and colchicine sensitive. *In vitro* studies on the association of tubule subunits derived from detergent-treated sea urchin sperm flagella demonstrated neither of these properties, although microtubules were observed (Stephens, 1968). In studies on the purified microtubule protein from porcine brain tissue, isolated subunits aggregated to form beaded and linear structures. This aggregation process was temperature dependent, colchicine inhibitable, and nucleotide and protein specific; however, the aggregates were not microtubules (Borisy *et al.*, 1972).

Recently, conditions were established for the temperature-induced formation of microtubules *in vitro* (Weisenberg, 1972; Borisy and Olmsted, 1972). As determined by electron microscopy, viscometry, and sedimentation, this reaction was sensitive to both low temperature and colchicine (J. B. Olmsted *et al.*, manuscript in preparation). This paper describes the use of viscometry to define the optimum conditions for preparing extracts of porcine brain tissue in which polymerization is rapid, and the formed tubules are stable. In addition, these experiments have broadly defined some of the basic characteristics of the *in vitro* assembly reaction.

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Materials and Methods

Preparation of Extracts. Porcine brain was obtained within 2 hr of slaughter (courtesy of Oscar Mayer and Co., Madison, Wis.), and chilled on ice, and the meninges and superficial blood vessels were removed; all subsequent operations were then carried out at 0°. One part of brain cortex (grams wet weight) was homogenized in a glass homogenizer with 1.5 volumes (ml) of PEG buffer[1] (100 mM Pipes–1 mM EGTA–2.5 mM GTP, adjusted with 5 N NaOH to a pH of 6.94 at 23°) using five strokes of a motor-driven Teflon pestle operated at 2000 rpm. For some experiments (see Results and figure legends), brain was homogenized in PE buffer (100 mM Pipes–1 mM EGTA (pH 6.94)) which contained other compounds. The homogenate was centrifuged at 25,000*g* (20,000 rpm for 1 hr, Spinco Type 65 fixed angle rotor, 9.0-ml polycarbonate tubes) and the supernatant fraction (extract) was used for subsequent experiments. The protein concentration of the extract (range, 10–12 mg/ml) was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Extracts prepared from fresh brains polymerized to a greater extent than extracts prepared from brains obtained several hours after slaughter. In addition, the ability of the extracts to polymerize decreased during the day, and extracts were therefore used within 3 hr of preparation.

Viscometry. Ostwald capillary viscometers (Cannon-Manning semimicro viscometer, Type 100, Cannon Instrument Co., State College, Pa.) were immersed in a large water bath regulated at 37.0 ± 0.1°, and outflow times for samples were measured using stopwatches calibrated to 0.2 sec. The outflow time at 37° for 0.6 ml of PEG buffer was determined for each viscometer, and the values ranged from 51.0 to 56.0 sec. For experiments in which the incubation temperature was altered (see Results), viscometers were recalibrated at the appropriate temperature. To obtain experimental data, 0.6 ml of extract prepared at 0° was placed in a viscometer equilibrated at 37°, and viscosity development was followed as a function of time of incubation. Temperature equilibration was rapid, occurring within the first minute of incubation. Measurements on extracts prepared under various conditions were made in the same water bath, and kinetic data for up to 12 polymerization reactions were collected simultaneously. Data are expressed as specific viscosity, η_{sp}, which was calculated in terms of outflow times of buffer (OT_b) and extract (OT_e) by the equation

$$\eta_{sp} = (OT_e - OT_b)/(OT_b)$$

Variations in the kinematic viscosity introduced by slight differences in density were negligible and therefore density corrections were not normally made.

Electron Microscopy. Samples of extracts were prepared for electron microscopy using negative staining procedures. A 5-μl sample was placed for 30 sec on a 400 mesh grid coated with formvar and carbon. The sample was then displaced successively with 4 drops of each of the following solutions: 1-mg/ml cytochrome *c;* distilled water; 1% uranyl acetate. The excess uranyl acetate was drawn off with filter paper, and the air-dried samples were examined with a Philips 300 microscope operated at 80 kV.

[1] Abbreviations used are: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)tetraacetic acid; PE buffer, 100 mM Pipes–1 mM EGTA, adjusted to pH 6.94 at 23°; PEG buffer, PE buffer including 2.5 mM GTP; GMPPCP, β,γ-methylene guanosine triphosphate.

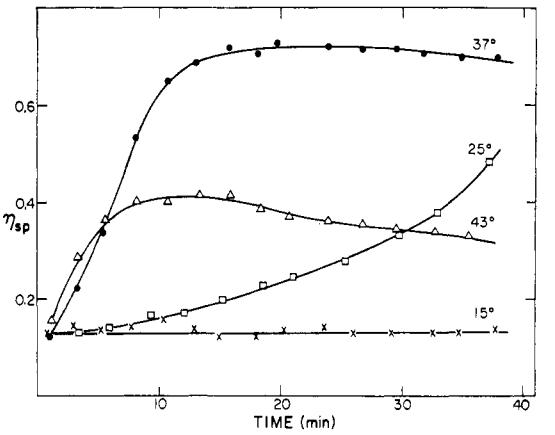

FIGURE 1: Temperature dependence of polymerization kinetics. Viscosity development in aliquots of an extract incubated at 15 (×), 25 (□), 37 (●), and 43° (△). Protein concentration, 10.3 mg/ml.

Results

General Considerations on Experimental Procedure. Extracts were prepared using a variety of conditions in order to establish the optima for the rate and extent of microtubule formation *in vitro.* Tubulin comprised approximately 25–30% of the total protein in these extracts (J. B. Olmsted *et al.*, manuscript in preparation). Although some variability was observed among preparations made on different days, this was minimized by homogenizing the brain immediately upon delivery, and by standardizing the time of preparation such that viscosity measurements were always made within 1.5 hr of obtaining the extract. Extracts held for more than 4 hr developed low viscosity levels. For any of the parameters described in the following section, simultaneous measurements were made on material derived from the same preparation, and the rates and extents of polymerization are therefore internally comparable. Samples from all experiments were examined by electron microscopy and, invariably, high viscosity levels corresponded to large numbers of long microtubules. Qualitatively, the kinetics of viscosity development correlated with observations of both increased number and length of microtubules; however, a precise quantitative relationship between these parameters has not yet been determined.

Temperature Optima. To define the optimal temperature of polymerization, aliquots of an extract were incubated at a variety of temperatures, and the kinetics of viscosity development were followed (Figure 1). At 15°, no viscosity was developed, nor were any microtubules observed. At 25°, polymerization initially proceeded at a low rate but then increased steadily and ultimately attained a high plateau level (not shown in Figure 1). Viscosity developed more rapidly at 37° and a plateau was reached after 15 min. The initial rate of polymerization was higher at 43° than at 37°; however, the maximal viscosity value was higher and more stable at 37°.

To define the temperature optimum for the maximum amount of polymerization, regardless of the rate of viscosity development, data were expressed as the maximum viscosity attained *vs.* the temperature of incubation (Figure 2, curve A). These data indicated a temperature optimum of 27° for maximal polymerization. Although possible, we thought it unlikely that the *in vitro* reaction would have an optimum 10° below the temperature at which polymerization would normally occur in the source tissue. Since it was possible that the high viscosity values at the different temperatures were not comparable due to inter- and intratubule interactions, experi-

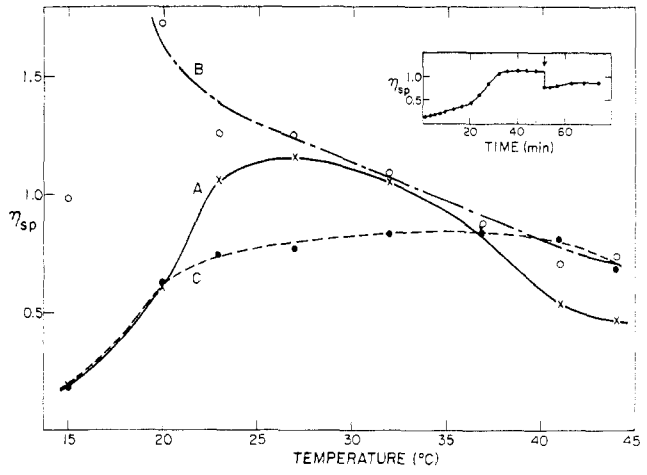

FIGURE 2: Temperature optimum for polymerization. See text for detailed explanation of curves. Curve A: plateau value of η_{sp} attained at incubation temperature (T) (×). Curve B: temperature dependence of plateau η_{sp} value for a given amount of polymer (○). The 15° point is low because of rapid depolymerization occurring at this temperature. Curve C: temperature optimum for the formation of polymer (●). The insert shows the time course of viscosity development in a shift experiment where polymerization occurred at 27° and the temperature was then shifted to 37° at the time denoted by the arrow. Protein concentration, 10.4 mg/ml.

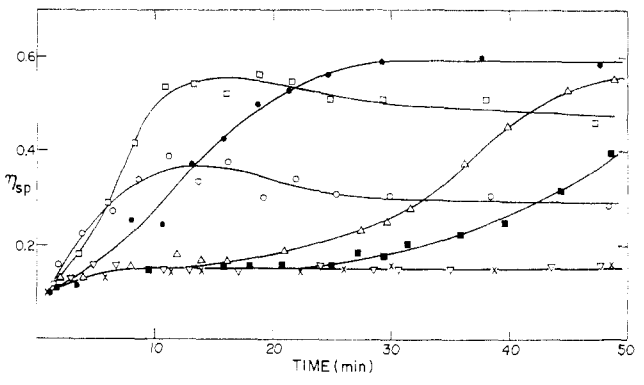

FIGURE 3: pH dependence of polymerization kinetics. Extracts were prepared by homogenizing brain in PEG buffer adjusted to the following pH values at 23°: 5.24, 5.96, 6.70, 6.94, 7.15, 7.35, 8.78. The corresponding pH at 23° of the final extracts, after adjusting to a protein concentration of 8.6 mg/ml, were, respectively, 5.86 (×), 6.11 (○), 6.67 (□), 6.90 (●), 7.05 (△), 7.18 (■), 7.59 (▽).

ments were carried out to determine the dependence of specific viscosity on temperature for a fixed amount of polymerized material. Aliquots of an extract were incubated at 37° until the maximum level was reached; the samples were then shifted to various temperatures between 15 and 45°, and the specific viscosity was measured. The initial specific viscosity values at the second temperature are plotted in curve B in Figure 2. At temperatures greater than 25°, the viscosity values at the second temperature remained stable; these data therefore accurately reflect the temperature dependence of specific viscosity for a given amount of polymerized material. Over the temperature range of 25–40°, η_{sp} for a given amount of polymer was approximately inversely proportional to the temperature, and the temperature-dependence curve (curve B) followed that of the apparent optimum curve for polymerization (curve A). After shifts to lower temperatures (less than 20°), the kinetic data showed a rapid decline in viscosity levels; this indicated tubule depolymerization was occurring. Therefore, these measurements reflected the opposing factors of increased η_{sp} values due to the lower temperatures and decreased amount of polymer due to disassembly.

To determine the actual optimum temperature for the formation of the maximum amount of polymer, the η_{sp} values for the plateau levels of the extracts incubated at various temperatures were all measured at a single temperature. In these experiments, aliquots of an extract were incubated at the different temperatures until the maximal specific viscosity level was reached; the samples were then shifted to 37° and the viscosity was measured. The initial points recorded after the shifts to the second temperature (37°) are shown in curve C of Figure 2, and the curve depicts the maximum amount of polymer formed at a given temperature. Over the temperature range of approximately 30–40°, essentially similar amounts of microtubules, as reflected by the specific viscosity values, were formed. At temperatures between 20 and 30°, there was a gradual decline in the amount of polymer formed as the temperature decreased, and at 15° no polymerization had occurred. At temperatures greater than 40°, polymerization was below the maximal value. Analysis of the kinetic curves obtained after the shift to the second temperature indicated that extracts originally incubated at temperatures lower than 30° continued to polymerize until the maximal level of extracts initially incubated at 37° was attained. For example, the insert in Figure 2 shows a shift experiment in which the sample was first incubated at 27°, and then shifted to 37° at 50 min. In the case of the extract originally incubated at 15° in which no polymerization had occurred, viscosity developed upon shifting to 37° with the same kinetics as observed for the aliquot initially incubated at 37°. In determining the temperature at which to follow polymerization, 37° was used because of the maximal and relatively stable plateau level.

pH Conditions. To determine the optimum pH at which to prepare extracts, brain tissue was homogenized in PEG buffer adjusted with 5 N NaOH or HCl to a range of pH values. After adjusting the samples to the same protein concentration, the resultant pH of an aliquot of the extract was measured at room temperature. The protein solution itself had a large buffering capacity (see legend, Figure 3) and dilution to obtain a uniform protein concentration had no detectable effect on the pH of the final preparation. As shown in Figure 3, no assembly occurred at extreme pH values (5.86, 7.59). At slightly acid pH (6.11, 6.67, 6.90), the initial rate of polymerization was rapid, although both plateau levels and stability varied. At slightly alkaline pH (7.05, 7.18), the initial rate of polymerization was low, although viscosity developed more rapidly with continued incubation. In terms of the maximal level of viscosity attained at each pH (Figure 4), the pH optimum for the reaction was between 6.8 and 7.0, with a more gradual decline in plateau levels on the acidic side of the optimum. Taking into account both the value and stability of the maximum, extracts were prepared for all subsequent experiments in PEG buffer at pH 6.94. Based on a temperature coefficient of −0.0085 pH unit/deg for Pipes buffer (Good *et al.*, 1966), the lowest possible pH value for such an extract at 37° would be approximately 6.8.

GTP Concentration. Guanine nucleotides are usually bound to microtubule protein isolated from various sources (Stephens *et al.*, 1967; Shelanski and Taylor, 1968; Yanagisawa *et al.*, 1968; Bryan, 1972), and inclusion of GTP in isolation media for microtubule subunits has been shown to both stabilize the protein (Weisenberg *et al.*, 1968; Wilson, 1970; Ventilla *et al.*, 1972) and promote *in vitro* aggregation (Borisy *et al.*, 1972). In addition, equilibrium dialysis studies using ^{3}H-GTP have indicated that the protein is capable of binding 2 mol of

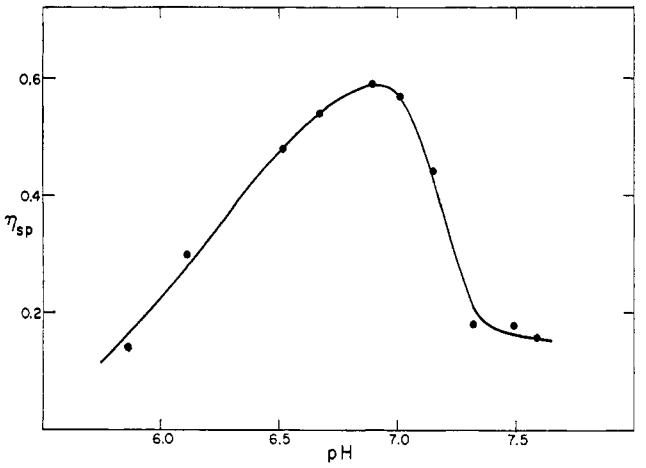

FIGURE 4: pH optimum for polymerization. Extracts were prepared as in Figure 3 and incubated at 37°. η_{sp} values represent the maximum viscosity reached, regardless of the stability of the level.

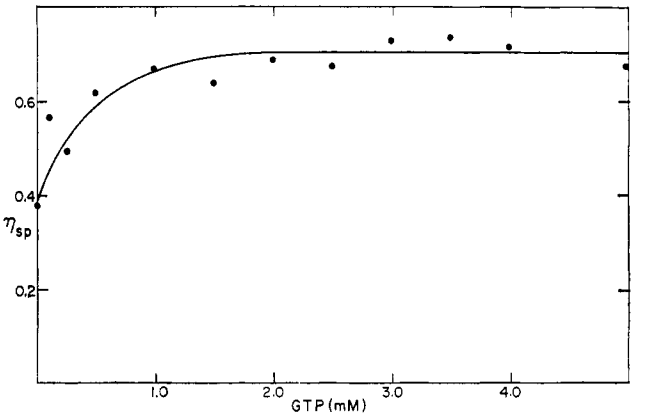

FIGURE 6: Dependence of maximum viscosity value on GTP concentration. Extracts were prepared as in Figure 5. η_{sp} values represent the maximum value reached irrespective of the stability of the value.

guanine nucleotide per dimer molecule; one of these is tightly bound, whereas the other is rapidly exchangeable (Weisenberg *et al.*, 1968; Berry and Shelanski, 1972). Therefore, a range of GTP concentrations was investigated in analyzing the optima for preparation of the extracts. Portions of porcine brain were homogenized in PE buffer (pH 6.94) containing different concentrations of GTP. The protein concentrations of the extracts were then adjusted to the same value with the appropriate buffer, and polymerization at 37° was followed (Figure 5). Some polymerization occurred in extracts which were prepared in the absence of GTP. Preparations of this type from several experiments varied considerably in rate, plateau, and stability of the plateau (see Figure 7 (○) for comparison); this might be due to differing amounts of residual nucleotide bound to the microtubule subunits in these preparations. At concentrations of GTP less than 3.0 mM (shown: 1.0, 2.5), initial rates of polymerization were rapid and the plateaus were reached in 10–15 min; plateau levels at the higher end of the concentration range were more stable. At GTP concentrations greater than 3.0 mM (shown: 4.0 mM), the initial rate of polymerization was slower, although the final plateau level approximated that of the extract prepared with 2.5 mM GTP. As shown in Figure 6, extracts prepared with concentrations of GTP greater than 2.0 mM attained similar maximal plateau levels. At lower concentrations, maximal levels were lower, and as shown from the kinetic curves, the plateaus were less stable. Therefore, to optimize both for rate of polymerization and stability of plateau, the concentration of GTP chosen to prepare extracts was 2.5 mM.

Nucleoside Specificity. The effect of nucleoside triphosphates other than GTP on the polymerization reaction was also examined. Homogenates were prepared in PE buffer which contained 2.5 mM concentrations of the appropriate compound, and the relative polymerization in the resultant extracts was determined (Figure 7). Extracts made with GTP developed viscosity at both the maximal rate and to the greatest plateau as compared with extracts made with the other nucleoside triphosphates. Samples prepared with CTP and UTP attained approximately the same viscosity level as extracts prepared in the absence of nucleoside triphosphate, although the initial rates varied. In contrast, little polymerization occurred in extracts prepared with ATP; microtubule assembly in this extract was therefore reduced below the level observed in an extract prepared in the absence of nucleotides.

In contrast to the effect of including various nucleotides in the homogenization buffer, different results were obtained if the nucleotides were added immediately before incubation to extracts prepared in PE buffer alone. Under these conditions,

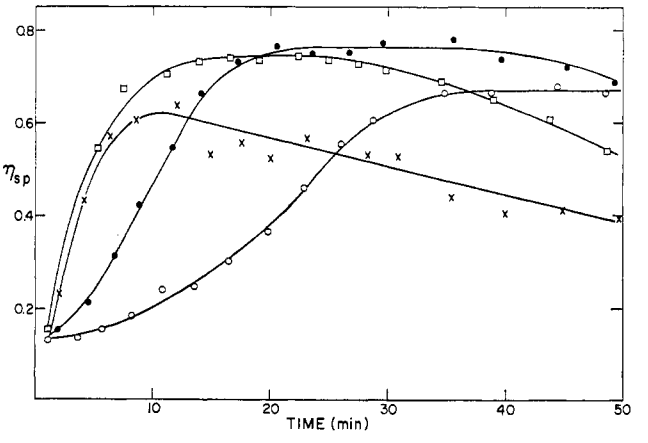

FIGURE 5: Effect of GTP concentration on polymerization kinetics. Extracts were prepared as described in the text, and all adjusted to a final protein concentration of 10.5 mg/ml. Millimolar GTP concentration: 0 (×), 1.0 (□), 2.5 (●), 4.0 (○).

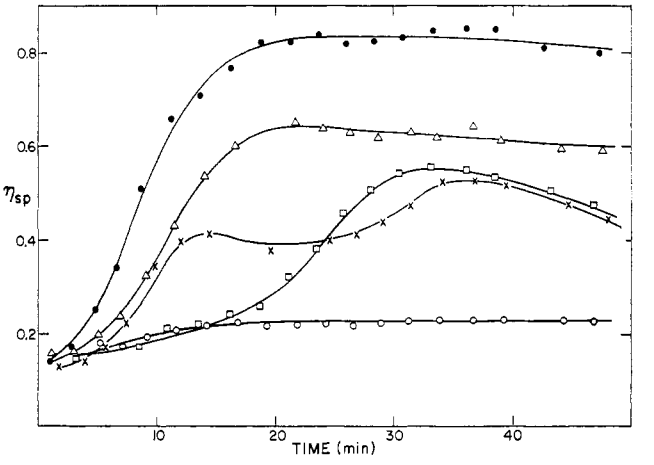

FIGURE 7: Effect of nucleoside triphosphates on polymerization. Extracts were prepared in PE alone (×) or PE containing 2.5 mM ATP (○), CTP (△), GTP (●), or UTP (□). Final protein concentration, 8.9 mg/ml.

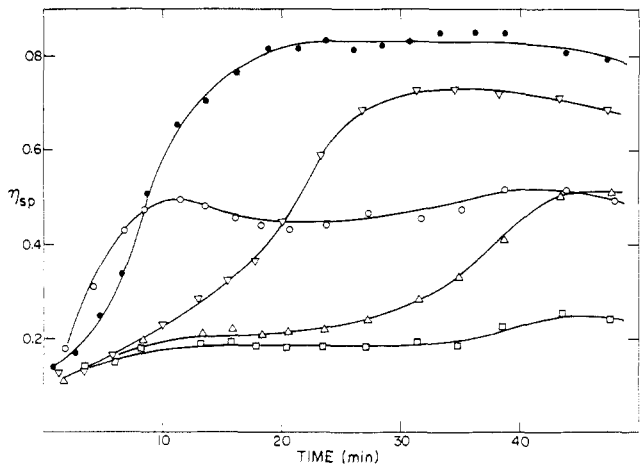

FIGURE 8: Effect of guanosine nucleotides on polymerization. Extracts were prepared in PE buffer containing 2.5 mM P_i (○), PP_i (△), GMP (□), GDP (▽), or GTP (●). Data for this figure were obtained simultaneously with those shown in Figure 7. Protein concentration, 8.9 mg/ml.

GTP, CTP, UTP, and ATP all promoted polymerization to a similar extent, and to a level greater than that observed in extracts without added nucleotide. This latter result is consistent with the observation of Weisenberg (1972) on the similar effectiveness of ATP and GTP in promoting the polymerization of partially purified microtubule protein. We have no explanation for the different responses of these two types of preparations to nucleotides, although similar results were also obtained in studies on the *in vitro* aggregation of purified microtubule protein (Borisy *et al.*, 1972, and unpublished results).

Since extracts prepared with GTP gave both the maximal rate and level of polymerization, the effect of making extracts with other guanosine nucleosides was also examined. In this series, extracts made with GTP again gave both maximal rates and plateau levels (Figure 8). In contrast, negligible viscosity development occurred in the presence of GMP; similar results (not shown) were obtained with extracts made with cyclic GMP or cyclic AMP, or the nonhydrolyzable analog of GTP, GMPPCP. The effects of probable reaction products from hydrolysis, namely, inorganic phosphate (P_i) and pyrophosphate (PP_i), were also tested. The presence of 2.5 mM P_i

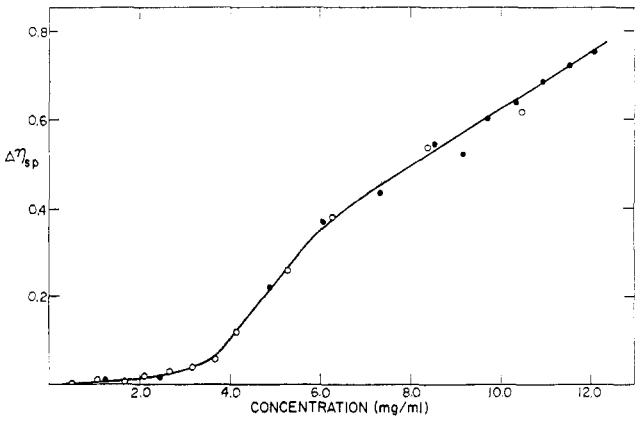

FIGURE 9: Concentration dependence of polymerization. Extracts were diluted with PEG buffer immediately before polymerization. Initial protein concentration of extract: 12.2 mg/ml (●), 10.5 mg/ml (○). Data are expressed as change in η_{sp} (plateau η_{sp} − initial η_{sp}).

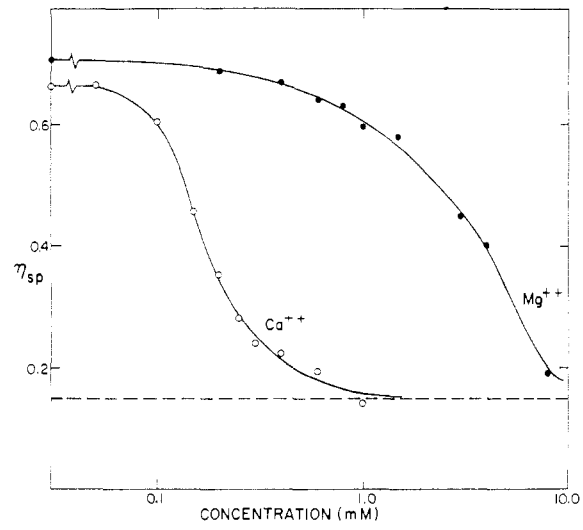

FIGURE 10: Effect of divalent cations on polymerization. Extracts were prepared as described in Materials and Methods except EGTA was excluded from the homogenization buffer. Immediately before incubation, aliquots of an extract were made to different ion concentrations with either $CaCl_2$ (○) or $MgCl_2$ (●) in EGTA-free buffer. Data are expressed as the plateau level reached, and the dashed line indicates the initial viscosity level of the extract. Protein concentration, 11.6 mg/ml.

(as NaH_2PO_4) in the extract resulted in polymerization essentially identical with that for an extract prepared in PE buffer alone (see Figure 7 (○) for comparison). In contrast, extracts prepared with PP_i showed a pronounced lag in polymerization, although the final maximum was similar to that for the P_i extract.

Firm conclusions concerning the specific nucleotide dependence of polymerization in crude extracts are unwarranted because of the unknown endogenous concentrations of nucleotides and the unknown activities of kinases, hydrolases, and transphosphorylases. However, the current data do suggest that the binding of GTP and the hydrolysis of the terminal phosphate groups are probably involved in microtubule assembly.

Concentration Dependence. To determine the protein concentrations in the extract at which polymerization would occur, experiments were carried out in which aliquots of an extract were diluted with buffer. Figure 9 demonstrates that at total protein concentrations in the extract greater than 4.0 mg/ml, the change in specific viscosity (plateau level minus initial value) was approximately proportional to the protein concentration. However, at concentrations lower than 4.0 mg/ml, little or no polymerization occurred. Electrophoretic analysis of extracts on sodium dodecyl sulfate-containing acrylamide gels indicated that tubulin comprised approximately 25% of the total protein in the extract (J. B. Olmsted *et al.*, manuscript in preparation). Therefore the critical concentration corresponds to an estimated tubulin content of approximately 1.0 mg/ml. Similar types of polymerization exhibiting an abrupt transition between monomeric and polymeric states at some critical concentration have been observed for the *in vitro* assembly of tobacco mosaic virus protein (Caspar, 1963; Durham *et al.*, 1971) and actin (Oosawa and Kasai, 1962).

Ionic Effects. It has been demonstrated that calcium is a potent inhibitor of microtubule polymerization (Weisenberg, 1972). In view of this finding, and the general expectation that polymerizing systems are sensitive to ionic conditions, the

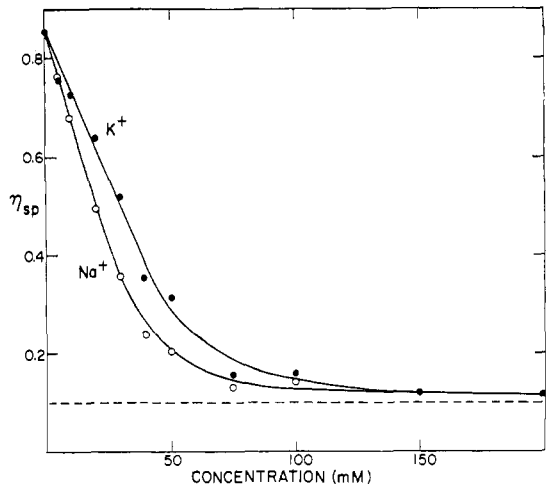

FIGURE 11: Effect of monovalent cations on polymerization. NaCl (○) or KCl (●) was added to extracts immediately before incubation. Data are expressed as the maximum plateau level reached in the presence of the monovalent cation. Dashed line indicates the initial viscosity level of the extract. Protein concentration, 10.9 mg/ml.

effects of various concentrations of both mono- and divalent cations on microtubule polymerization were examined. At a concentration of 1 mM, calcium completely inhibited polymerization in extracts prepared in the absence of EGTA; in addition, the same concentration added at any time during the course of polymerization caused an immediate loss of viscosity and the disruption of microtubules (J. B. Olmsted *et al.*, manuscript in preparation). When added immediately before incubation, lower concentrations of Ca^{2+} depressed the final plateau level attained, and viscosity development was half-maximal at a concentration of 0.2 mM (Figure 10). In contrast, Mg^{2+} had similar effects but at concentrations approximately ten times those of Ca^{2+}. Half-maximal plateau levels were attained with 4.0 mM Mg^{2+}, and total inhibition occurred at concentrations greater than 10 mM. The effects of two monovalent cations, Na^+ and K^+, on polymerization are shown in Figure 11. Half-maximal plateau levels were reached with either Na^+ or K^+ at concentrations of 30–40 mM, and total inhibition occurred at concentrations greater than 150 mM. Concentrations of 100 mM Na^+ and K^+ added to extracts which had been polymerized to the maximum level caused a rapid depolymerization similar to that observed for Ca^{2+} induced depolymerization. The kinetic curves in all these experiments indicated that both mono- and divalent cations depressed the reaction uniformly; *i.e.*, both rate and plateau levels were affected. It should be noted that these data define general levels of inhibition, since the contribution of intra- and extracellular ions from the brain tissue is unknown.

Effect of Microtubule Stabilizing Agents. Studies on the mitotic spindle have demonstrated that some reagents (D_2O, hexylene glycol, other alcohols) cause increases of two- to threefold in spindle briefringence (Inoué and Sato, 1967; Rebhun and Sawada, 1969), theoretically by shifting the equilibrium toward polymer formation. Therefore, the effect of D_2O on the *in vitro* assembly process was examined to determine if increased polymerization of tubules would occur. Aliquots of an extract were diluted by up to 50% with PEG buffer made in either water or D_2O (Figure 12). In the presence of increasing concentrations of D_2O, there was a corresponding increase in the initial rate of polymerization. Higher concentrations of D_2O also resulted in an increase in the

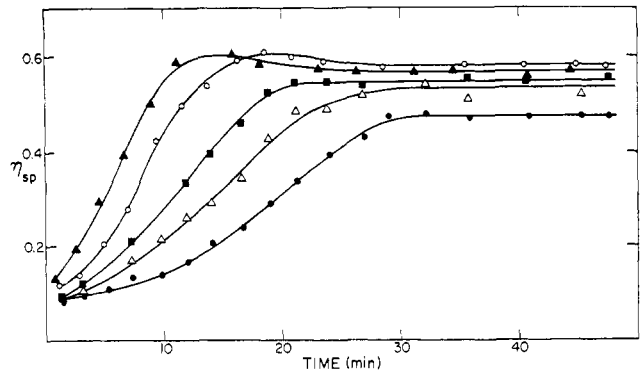

FIGURE 12: Effect of D_2O on polymerization. An extract prepared in PEG buffer was diluted by 50% with various proportions of PEG buffer made either in water or D_2O. The aliquots were then incubated at 37° immediately after dilution. Final % D_2O concentration: 0 (●), 10 (△), 20 (■), 30 (○), 50 (▲). Protein concentration, 6.2 mg/ml.

maximal plateau level; however, the viscosity developed in 30% D_2O was only 25% higher than the value for the sample polymerized in PEG buffer alone. At a concentration of 50% D_2O, the extent of viscosity development was slightly below the level obtained at 30% D_2O.

Experiments were also carried out with hexylene glycol, which has been shown to cause a twofold increase in mitotic spindle birefringence when added at concentrations up to 1.0 M (Rebhun and Sawada, 1969). At concentrations of hexylene glycol between 0.2 and 1.0 M, viscosity increased rapidly and microtubules were formed (data not shown). However, hexylene glycol also caused the formation of turbidity in the extracts. The reliability of viscosity readings in the presence of this reagent was therefore questionable, and further work is required to determine the mechanism of action of this substance.

Colchicine Sensitivity of Polymerization. Colchicine has been demonstrated to bind to microtubule subunits (Borisy and Taylor, 1967a,b; Wilson and Friedkin, 1967; Wilson and Bryan, 1973), and it is thought that this drug disrupts tubule assembly by causing a shift in the monomer–polymer equilibrium (Inoué and Sato, 1967). In viscosity studies, it has been shown that 100 μM colchicine will completely inhibit development of viscosity in extracts, but will cause only the partial depolymerization of formed tubules (J. B. Olmsted *et al.*, manuscript in preparation). In the present study, the concentration range of colchicine which would initially block polymerization was investigated. Kinetic data demonstrated that colchicine inhibition occurred by depression of the entire reaction curve. As expressed in terms of the effect on final plateau levels (Figure 13), colchicine had little effect on polymerization at concentrations of less than 0.05 μM, caused half-maximal inhibition at 0.5 μM, and was completely inhibitory at concentrations greater than 1 μM. From this result it is estimated that only a fraction of the theoretical number of colchicine-binding sites need to be complexed for complete inhibition to occur (see Discussion).

Discussion

The conditions under which microtubule subunits in porcine brain extracts polymerize have been studied by viscometry. The initial use of the extract to examine these parameters was pursued for two reasons. Primarily, the optimum conditions could be used in the development of an efficient purification

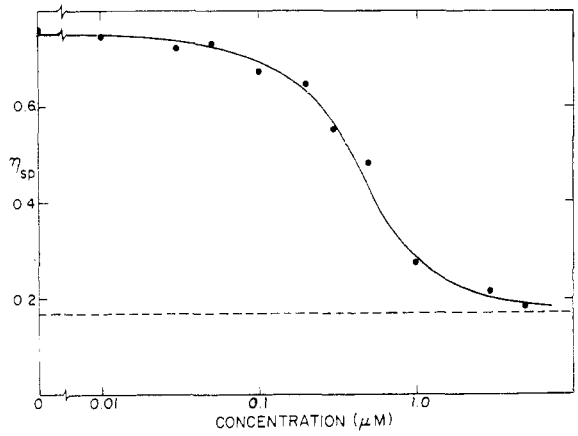

FIGURE 13: Colchicine inhibition of polymerization. Aliquots of an extract were mixed with different concentrations of colchicine immediately before incubation. Data are expressed as the maximal plateau level reached. Dashed line indicates the initial viscosity level of the extract. Protein concentration, 12.1 mg/ml.

scheme in which competent microtubule subunits were isolated by repeated cycles of assembly and disassembly (J. B. Olmsted *et al.*, manuscript in preparation). In addition, a comparison of polymerization in crude extracts and purified preparations might reveal factors in the extracts involved with the regulation of microtubule assembly. Viscometry has been demonstrated to be a useful method for the characterization studies, since it enables both the rapid measurement of polymerization as well as the simultaneous determination of numerous parameters. The observation that viscosity measurements reflect biological properties of microtubule polymerization has been shown by the sensitivity of viscosity levels to both temperature and colchicine (J. B. Olmsted *et al.*, manuscript in preparation). Therefore, as compared with electron microscopy or light scattering (Weisenberg, 1972; Borisy and Olmsted, 1972; Shelanski *et al.*, 1973), viscometry has proved to be a rapid, sensitive, and quantitative method for analyzing assembly conditions.

The assembly reaction has many of the properties characteristic of the formation of microtubules *in vivo*. For example, assembly proceeded under nearly physiological conditions, with neither extreme pH, temperature, or ionic strength being required. In addition, the kinetics of the reaction, in which maximum polymerization occurs within 15–20 min at 37° and depolymerization within 10 min at 0°, were similar to those observed for the appearance and dissolution of the spindle in intact oocytes (Inoué and Sato, 1967). Microtubule assembly *in vitro* is also endothermic, a property of spindle formation *in vivo* which has been studied using birefringence measurements (Inoué and Sato, 1967; Stephens, 1973). Therefore, in terms of morphology, kinetics, drug sensitivity, and temperature dependence, microtubule polymerization *in vitro* is similar to the *in vivo* assembly of microtubules in the mitotic spindle.

Colchicine binding has been used as a specific means of identifying tubule subunits during purification (Weisenberg *et al.*, 1968, Bryan and Wilson, 1971); in addition, the property has been considered indicative of the native condition of the protein (Wilson, 1970). Since colchicine inhibits microtubule assembly and also binds to the subunits (Borisy and Taylor, 1967a; Wilson and Friedkin, 1967), it seemed probable that the optimum conditions for both preparation of extracts and polymerization would resemble the optima for the drug-binding reaction. On comparison, the pH optimum (6.7–6.9) and temperature optimum (37°) for maximal assembly were the same as those observed for colchicine binding (Borisy, 1966; Wilson, 1970), and at 0°, neither assembly nor colchicine binding occurred. Similarly, inclusion of GTP, which both maximized and stabilized viscosity levels in microtubule polymerization, also stabilized the colchicine-binding activity of isolated subunits (Weisenberg *et al.*, 1968; Wilson, 1970; Ventilla *et al.*, 1972). In view of the inhibitory properties of colchicine, these similarities between the optima for drug binding and assembly suggest that the colchicine-binding site may spatially overlap one of the bonding sites on the microtubule subunit involved in polymerization. This hypothesis is consistent with the observation that colchicine binds only to subunits and not to intact microtubules (Wilson and Meza, 1973).

The sensitivity of the *in vitro* assembly reaction to colchicine is striking, with concentrations of colchicine as low as 1.0 μM completely inhibiting tubule formation. As estimated from the tubulin content of the extract (approximately 25% of the total protein), the binding constant of colchicine ($K = 1 \times 10^6$ l./mol at 37°) (Borisy and Taylor, 1967a), and the stoichiometry of colchicine binding (1 mol/110,000 g of tubulin) (Weisenberg *et al.*, 1968; Bryan, 1972), less than 4% of all microtubule subunits would be bound to colchicine in order for polymerization to be totally inhibited. A similar low percentage of sites complexed with colchicine was the minimum required to block mitosis in cultured cells (Taylor, 1965). These results cannot be explained simply in terms of the commonly suggested mechanism for colchicine inhibition. Colchicine has been postulated to prevent microtubule assembly and mitotic spindle formation by binding to the tubule subunits (Borisy and Taylor, 1967; Wilson and Friedkin, 1967) and shifting the subunit–polymer equilibrium toward the monomeric state (Inoué, 1952). This mechanism predicts that colchicine would be effective at concentrations where the majority of subunits are complexed. However, the present results indicate that inhibition by colchicine at low levels of complex formation may have to be explained by another scheme. For example, one possibility is that colchicine may directly interfere with microtubule elongation by binding to the terminal subunit and preventing further addition of subunits. Alternatively, a colchicine subunit complex could add to the growing end of the tubule to inhibit polymerization.

The effect of nucleosides on microtubule assembly was examined in the course of determining the optimum conditions for extract preparation. As had been observed in studies on the *in vitro* aggregation of isolated microtubule subunits (Borisy *et al.*, 1972), GTP promoted maximal polymerization. In terms of the relative effects of the guanosine phosphates, the maximal levels developed with GTP suggest that the hydrolysis of terminal phosphate groups may be involved in the assembly process. However, GTP is apparently not required for microtubule assembly in solutions containing 1 M sucrose or 4 M glycerol, although polymerization under these conditions occurs very slowly (5 hr) (Shelanski *et al.*, 1973). Although the relationship of this finding to the mechanism of tubule assembly *in vivo* is not clear, analogous results have been obtained in studies on actin polymerization. Globular actin is isolated with 1 mol of ATP bound per mole of subunit, and the nucleotide is hydrolyzed to ADP upon the formation of fibrous actin (Szent-Györgyi, 1951). However, in sucrose solutions, actin does not require ATP for polymerization, although the rate of assembly is low (Kasai *et al.*, 1965). Even though polymerization in sucrose or glycerol solutions is not physiological, these observations have led to the suggestion that ATP is a kinetic

regulator of actin polymerization (Oosawa and Kasai, 1971). Studies of purified material in which nucleoside phosphate binding and hydrolysis can be quantitatively assayed will be important in determining whether nucleosides have a regulatory function in microtubule polymerization.

The concentration dependence of microtubule formation resembled the type of dependence seen in studies on the *in vitro* assembly of purified actin (Oosawa and Kasai, 1962) and tobacco mosaic virus protein (Caspar, 1963). In these systems, an abrupt transition occurred between the monomeric and polymeric states at a critical concentration of subunits. This concentration dependence has been explained by the requirement for a cooperative interaction of subunits to take place before linear growth will occur. In the case of actin polymerization, it has been inferred that one turn of the actin helix (four subunits) must be formed for polymerization to continue (Oosawa and Kasai, 1962), and in tobacco mosaic virus assembly, a disk structure composed of 34 subunits is initially made (Caspar, 1963; Durham *et al.*, 1971). It has been suggested that nucleating sites may also be required in the *in vitro* assembly of microtubules (Borisy and Olmsted, 1972). Experiments on purified tubule protein will be useful in determining if an equilibrium involving these disk-like structures is related to the concentration dependence of microtubule polymerization.

In conclusion, this report has described the use of viscometry to establish the optimal conditions for the formation of microtubules *in vitro*. Various properties of the assembly process, such as nucleotide specificity and ion sensitivity, have been quantitatively characterized and suggest possibilities for the cellular regulation of polymerization. The optimal conditions have already been used to obtain purified material by repeated cycles of assembly and disassembly, and this material should prove valuable in elucidating some of the properties of microtubule polymerization.

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