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EFFECTS OF GLYCEROL ON MICROTUBULE POLYMERIZATION KINETICS[1]

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SUMMARY: Steady state and kinetic studies of polymerization of purified microtubule protein show little effect of glycerol on the steady state level of polymerization, as demonstrated by measurements of critical concentration. The rates of polymerization and depolymerization are slowed in the presence of glycerol. This data indicates that the stabilization of microtubules by high glycerol is largely a kinetic effect rather than a shift in equilibrium. However, the apparent critical concentration for microtubule polymerization from crude brain homogenate is substantially higher in the absence of glycerol, and glycerol appears to protect microtubule polymerization against the action of endogenous inhibitors.

Glycerol is frequently used in buffers for the preparation of microtubules or microtubule protein (1,2). The advantages of glycerol include its ability to prevent spontaneous denaturation of tubulin (3), the prevention of sulfhydryl oxidation (4) and the attenuation of the effects of microtubule polymerization inhibitors found in cell extracts (5). Its disadvantages, when used during preparation, include effects on the protein composition of the microtubule protein so obtained (6). A report that glycerol binds irreversibly to tubulin (7) has discouraged its general use, but recent work has demonstrated that radiochemical impurities in labelled glycerol account for the apparent binding, and glycerol itself does not modify tubulin irreversibly (8).

I have undertaken here a study of the effects of glycerol on microtubule polymerization, using the methods of kinetic analysis of Johnson and Borisy (9). The concentrations of glycerol used in microtubule preparation have ranged up to 50% by volume [ca. 6.7 *M*] (2) although values of 2.5 - 4 *M* are more common.

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Under such conditions, modifications of rates and equilibria must be expected, and are demonstrated below.

MATERIALS AND METHODS

Bovine brains were obtained from the Abattoir, Dept. of Animal Science, University of Guelph. Buffers and GTP (Product No. G5756) were from Sigma Chemical Company, St. Louis.

Microtubule protein was isolated from bovine cerebral cortex, following the procedure of Murphy and Hiebsch (10). Purification of the protein was carried through four cycles of assembly and disassembly. The buffer used contained 0.1 *M* 2-(N-morpholino)ethanesulfonic acid, 1m*M* $MgCl_2$ 1 m*M* ethylenebis(oxyethylenenitrilo)tetraacetic acid, 1 m*M* GTP, and pH was adjusted to 6.6 with NaOH. A second buffer, containing the above ingredients, was made in 12 *M* glycerol instead of distilled water. The glycerol concentration of samples was adjusted by appropriate dilution with 12 *M* glycerol buffer.

Protein concentration was determined by the method of Lowry (10). Microtubule protein, dehydrated in absolute ethanol and dried over P_2O_5, was used as primary standard, and bovine serum albumin as a secondary standard. Microtubule protein at 1.23 mg/ml gives similar colour intensity to 1.0 mg/ml bovine serum albumin. The molarity of tubulin was then determined from the tubulin content (83%) and based on a molecular weight of 110,000 g mol^{-1} (12). A microtubule protein protein sample colorimetrically equivalent to 1.0 mg/ml bovine serum albumin thus contains 9.3 μ*M* tubulin.

Tubulin activity was verified by a colchicine binding assay (13). Microtubule protein composition was analyzed by polyacrylamide gel electrophoresis (14). The preparation contained 83% tubulin, 15% high molecular weight protein and 2% non-specific protein in terms of intensity of staining by Coomassie Brilliant Blue R. Turbidity of polymerized microtubule solutions was measured in a Unicam SP1700A spectrophotometer equipped with an automatic cuvet changer. Polymerization and depolymerization were induced by temperature step up to 37° and step down to 0°. Rapid changes in temperature were achieved by maintaining separate water circulators at 37.3° and -2.5°. The supply to the cuvet could be rapidly switched between these sources as necessary. The temperature of the cuvet contents was monitored by a thermistor probe (Yellow Springs Instruments) and the circulator temperatures used gave 37° and 0° in the cuvet at steady state. Temperature changes were complete within 0.3° in 90 seconds, a delay easily accommodated by the lag time for polymerization. Condensation on cuvet windows was controlled by a continuous purge of the cuvet chamber with dry air.

RESULTS AND DISCUSSION

The turbidity of microtubule solutions has been shown to depend on mass concentration of microtubules with little dependence on length or number concentration (15). Nevertheless, absolute values of turbidity are dangerous to use for comparison purposes if solution conditions differ, since factors such as the refractive index of the medium and the refractive index increment

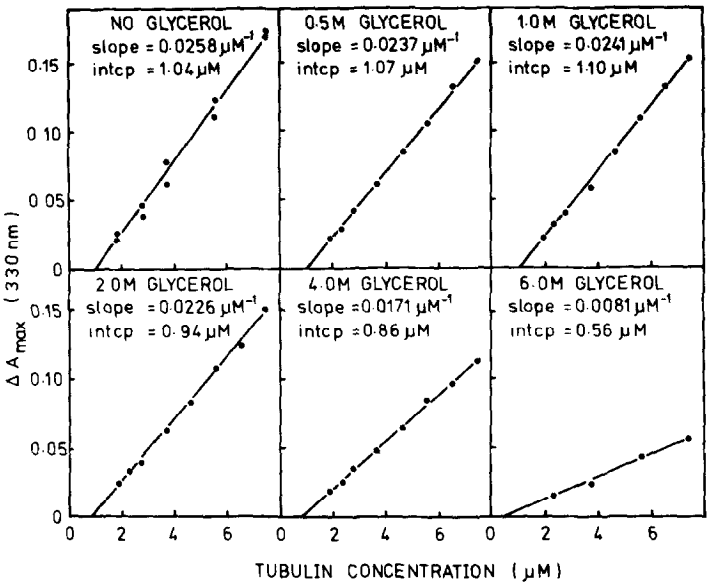

Figure 1. Turbidity developed by polymerized microtubules (ΔA_{max}): dependence on tubulin and glycerol concentrations. Path length = 1 cm. Up to 2.0 *M* glycerol, polymerization was 100% reversed on cooling to 0°. For 4.0 *M* and 6.0 *M* glycerol, depolymerization was too slow to observe to completion. Apparent critical concentration is the intercept on the concentration axis, and is uncorrected for inactive protein. Multiplication of intercept values by α (Table 1) gives the most reliable values for true critical concentration. The slopes are used to convert rates of turbidity change into rates of concentration change (Table 2).

due to the microtubules, which influence the turbidity (16), may also vary. Johnson and Borisy have shown how the critical concentration of tubulin, the limit below which no polymerization can occur, is equal to the reciprocal steady state constant for microtubule polymerization (9):

$$\text{critical concentration} = {}^{k_1}/_{k_2}$$

(k_2 = forward rate constant; k_1 = reverse rate constant). This value is independent of the absolute value of turbidity.

Critical concentrations were determined for a series of glycerol concentrations up to 6 *M* (Fig. 1). There is little change in critical concentration up to 4 *M*. Only in 6 *M* glycerol is the steady state displaced significantly to favor polymerization. The slopes of the critical concentration plots indicate the turbidity per unit mass of polymer, a factor that can be used to convert rates of turbidity change to rates of concentration change. The

TABLE 1

Centrifugation assay of microtubule polymerization in glycerol solutions

Glycerol concentration (*M*)	Supernatant from 2.32 μ*M* tubulin (μ*M*)	Supernatant from 9.3 μ*M* tubulin (μ*M*)	Critical concentration (μ*M*)	α fraction of active tubulin
0	1.15	2.12	0.83	0.86
2	1.11	1.95	0.83	0.88
4	0.98	1.76	0.72	0.89
6	0.78	1.38	0.58	0.92

Microtubule protein solutions at six concentrations from 2.32 μ*M* to 9.3 μ*M* tubulin were incubated at 37° for 30 min and were centrifuged at 100,000 x g_{max} for 1 hour (30°C). Supernatant concentrations were determined. The values quoted are based on linear regression analysis for the whole series, not single determinations. Correlation coefficients were better than 0.99 in each case. $(1-\alpha) = \Delta C_S/\Delta C_O$ where ΔC_S is the difference in supernatant concentrations and ΔC_O is the difference in total concentrations. Critical concentration is obtained from supernatant concentration by subtraction of the fraction of inactive protein present: $C_S - C_O(1-\alpha)$. Values are lower than for critical concentrations obtained by turbidity assays, because of the correction for inactive protein and because some non-microtubule material may have sedimented.

slopes may also be lowered if any appreciable fraction of the protein in the preparation is inactive in polymerization under the given conditions. To demonstrate that the low turbidity of 4 *M* and 6 *M* glycerol solutions of microtubules is probably due to refractive index effects, I have also measured polymerization by a centrifugation assay (9). Table 1 shows that the polymerization of microtubules in 6 *M* glycerol is as complete as in the absence of glycerol.

Glycerol changes the rates of polymerization and depolymerization, slowing both processes (Table 2). The effects are not a linear function of the glycerol concentration, and 0.5 *M* glycerol is only marginally different from a simple aqueous buffer. The most extreme effects are noted for the cold depolymerization of microtubules above 4 *M* glycerol. The slowness of depolymerization reactions in 4 *M* and 6 *M* glycerol did not allow me to follow these to completion, and it is possible that a stable steady state may be estab-

TABLE 2

Effects of Glycerol solutions on polymerization and depolymerization rate constants.

Glycerol *M*	k_2M min^{-1}	$k_{1,o°}M$ $\mu M\ min^{-1}$	$\langle \ell \rangle$ μm	C_o-C_c μM	M nM	k_2 $\mu M^{-1}min^{-1}$	k_1 min^{-1}	$k_{1,o°}$ min^{-1}
0	0.585	1.65	9.5	1.89	0.32	1830	1640	5160
0.5	0.433	1.08	9.6	1.4	0.24	1800	1660	4500
1	0.43	0.77	8.4	1.37	0.27	1590	1520	2850
2	0.236	0.196	7.7	1.50	0.32	740	610	610
4	0.129	0.0143	5.6	1.56	0.45	290	220	32
6	0.15	0.0042	4.0	1.81	0.74	203	104	5.6

k_2M is the apparent first order rate constant for polymerization (9).
$k_{1,0°}M$ is the apparent zero order rate constant for depolymerization at 0° (9).
$\langle \ell \rangle$ is the average microtubule length, determined by electron microscopy of glutaraldehyde fixed specimens: ca. 200 microtubules were measured for each sample.
C_o-C_c is polymerized tubulin; total minus critical concentrations.
M is microtubule number concentration: obtained from $\langle \ell \rangle$ assuming 8/13 nm per tubulin dimer (17). $M = 13(C_o-C_c)/8\langle \ell \rangle$.
k_2 is the forward rate constant for polymerization at 37°.
k_1 is the reverse rate constant for polymerization at 37°.
$k_{1,0°}$ is the rate constant for depolymerization at 0°.

lished at 0° for these solutions. For tubulin at a constant 9.3 μM, depolymerization is complete at 14° in the absence of glycerol, at 10° in 1 *M* glycerol and at 6° in 2 *M* glycerol.

Crude homogenates containing tubulin are readily stimulated to polymerize in warm glycerol solution, where they might not do so in aqueous buffer (18). This is illustrated in Figure 2. If the unpolymerized tubulin remaining in the supernatant is at steady state with microtubules, its concentration may be regarded as an apparent critical concentration. The high value of this apparent critical concentration is probably due to the effect of endogenous polymerization inhibitors such as polyanions (5,19). Since glycerol has relatively little effect on true critical concentration at the molarities used here, it is apparent that the primary effect of glycerol in crude mixtures is

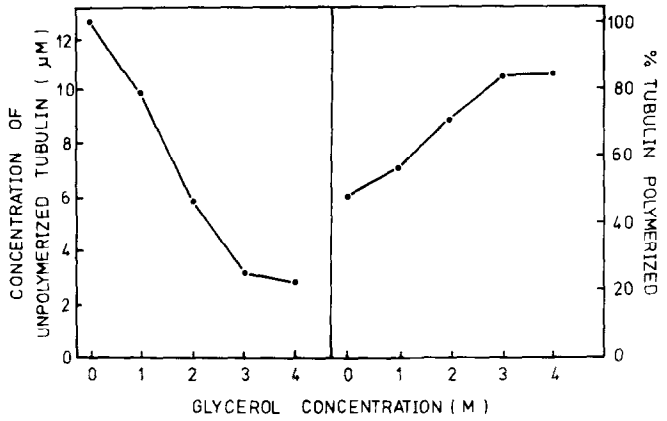

Figure 2. Effect of glycerol on microtubule polymerization in crude brain homogenate. Bovine cerebral cortex was homogenized in an equal volume of buffer (see Methods). After centrifugation at 40,000 x g_{max} for 1 h (4°), GTP was added (to 1 m*M*) to the supernatant (13 mg/ml total protein) and samples were prepared with different glycerol concentrations by addition of buffered 12 *M* glycerol. The samples were incubated at 37° for 30 minutes, and centrifuged at 100,000 x g_{max} for 1 h (30°). Supernatants and pellets were tested for colchicine binding activity (13). One nanomole of tubulin bound 15,320 cpm (0.53 nanomole) of [^{3}H] colchicine under the assay conditions used.

to attenuate that inhibition (5,18). Mammalian brain is tubulin rich, so concentrations present in the homogenate (23.8 μ*M* in this example) are well above the apparent critical concentration even in the absence of glycerol. Other tissues or cells may have tubulin at concentrations well below this so that the use of glycerol, at least in the first cycle of polymerization, is mandatory in such cases.

Several conclusions can be drawn from these results that could affect th design of experiments with microtubules. The advantages of stability of tubulin in high glycerol may be outweighed by the effects of the glycerol on reaction kinetics. I demonstrate here that dilute glycerol has marginal effects on polymerization kinetics, and thus a stock solution in high glycerol may be diluted for use without fear of deleterious effects. Secondly, polymerization of microtubules from crude solutions in 3 *M* - 4 *M* glycerol is much improved. One further application may also be of importance. Most studies on microtubules are made on structures reconstituted *in vitro*, and

attempts to isolate microtubules intact are more rare (2). Native microtubules may contain a distinct protein composition or organization that is not reproduced *in vitro*. However addition and loss of tubulin subunits is assymetrically distributed between microtubule ends, and there is a net tubulin flux through the microtubule (20). Thus, native microtubules, even when "stabilized" by the presence of assembly competent tubulin, will be speedily replaced by newly formed microtubules, without any net depolymerization being apparent. High glycerol concentrations, on the other hand, should stabilize native microtubules by slowing the steady state assembly-disassembly process. Conversely, any functional property of glycerol-stabilized microtubules dependent on the steady state tubulin flux will also be slowed, and may only be observed by diluting away the glycerol.

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