Lazarovici, P., & Yavin, E. (1985b) *Biochim. Biophys. Acta 812*, 532-542.
Lazarovici, P., Hochman, J., Menashe, M., Primor, N., & Zlotkin, E. (1982) *Arch. Toxicol. 51*, 167-173.
Lazarovici, P., Tayot, J. L., & Yavin, E. (1984) *Toxicon 22*, 401-413.
Lazarovici, P., Yanai, P., & Yavin, E. (1986) *J. Biol. Chem.* (in press).
Lee, G., Consiglio, E., Habig, H., Dyer, S., Hardegree, C., & Kohn, L. D. (1978) *Biochem. Biophys. Res. Commun. 83*, 313-320.
Lee, G., Grollman, E. F., Dyer, S., Beguinot, F., Kohn, L. D., Habig, W. H., & Hardegree, M. C. (1979) *J. Biol. Chem. 254*, 3826-3832.
Leskawa, K. C., & Rosenberg, A. (1981) *Cell. Mol. Neurobiol. 1*, 373-391.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Mellanby, J., & Whittaker, V. P. (1968) *J. Neurochem. 15*, 205-208.
Mellanby, J., & Pope, D. (1976) *Adv. Exp. Med. Biol. 71*, 215-229.
Mellanby, J., Van Heyningen, W. E., & Whittaker, V. P. (1965) *J. Neurochem. 12*, 77-79.
Morgan, I. G., Tettamanti, G., & Gombos, G. (1976) *Adv. Exp. Med. Biol. 71*, 137-150.
Ramos, S., Grollman, E. F., Lazo, P. S., Dyer, S. A., Habig, W. H., Hardegree, M. C., Kaback, H. R., & Kohn, L. D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4783-4787.
Rogers, T. B., & Snyder, S. H. (1981) *J. Biol. Chem. 256*, 2402-2407.
Schmitt, A., Dreyer, F., & John, C. (1981) *Naunyn-Schmiedeberg's Arch. Pharmacol. 317*, 326-330.
Sharom, F. J., & Grant, C. W. M. (1978) *Biochim. Biophys. Acta 507*, 280-293.
Soninno, S., Ghidoni, R., Marchesini, S., & Tettamanti, G. (1979) *J. Neurochem. 33*, 117-121.
Stoeckel, K., Schwab, M., & Thoenen, H. (1977) *Brain Res. 132*, 273-285.
Van Heyningen, W. E. (1974) *Nature (London) 249*, 415-417.
Van Heyningen, W. E., & Mellanby, J. H. (1968) *J. Gen. Microbiol. 52*, 447-454.
Wellhoner, H. H. (1982) *Rev. Physiol., Biochem. Pharmacol. 93*, 1-68.
Wong, M., Brown, R. E., Barenholz, Y., & Thompson, T. E. (1984) *Biochemistry 23*, 6498-6505.
Yavin, E. (1984) *Arch. Biochem. Biophys. 230*, 129-137.
Yavin, E., & Nathan, A. (1986) *Eur. J. Biochem. 154*, 403-407.
Yavin, E., Yavin, Z., Habig, W. H., Hardegree, M. C., & Kohn, L. D. (1981) *J. Biol. Chem. 256*, 7014-7022.
Yavin, E., Yavin, Z., & Kohn, L. D. (1983) *J. Neurochem. 40*, 1212-1219.

Direct Incorporation of Guanosine 5′-Diphosphate into Microtubules without Guanosine 5′-Triphosphate Hydrolysis

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Received January 28, 1986; Revised Manuscript Received July 17, 1986

ABSTRACT: Using highly purified calf brain tubulin bearing [8-^{14}C]guanosine 5′-diphosphate (GDP) in the exchangeable nucleotide site and heat-treated microtubule-associated proteins (both components containing negligible amounts of nucleoside diphosphate kinase and nonspecific phosphatase activities), we have found that a significant proportion of exchangeable-site GDP in microtubules can be incorporated directly during guanosine 5′-triphosphate (GTP) dependent polymerization of tubulin, without an initial exchange of GDP for GTP and subsequent GTP hydrolysis during assembly. The precise amount of GDP incorporated directly into microtubules is highly dependent on specific reaction conditions, being favored by high tubulin concentrations, low GTP and Mg^{2+} concentrations, and exogenous GDP in the reaction mixture. Minimum effects were observed with changes in reaction pH or temperature, changes in concentration of microtubule-associated proteins, alteration of the sulfonate buffer, or the presence of a calcium chelator in the reaction mixture. Under conditions most favorable for direct GDP incorporation, about one-third of the GDP in microtubules is incorporated directly (without GTP hydrolysis) and two-thirds is incorporated hydrolytically (as a consequence of GTP hydrolysis). Direct incorporation of GDP occurs in a constant proportion throughout elongation, and the amount of direct incorporation probably reflects the rapid equilibration of GDP and GTP at the exchangeable site that occurs before the onset of assembly.

Tubulin polymerization, both with and without MAPs,[1] generally requires GTP bound at the exchangeable nucleotide site (Weisenberg et al., 1968) of the protein. In the course of the reaction this GTP is hydrolyzed to GDP (Kobayashi, 1975). These observations have generated much experimental work and a number of intriguing models to elucidate the role of GTP hydrolysis in the formation and stability of microtubules [e.g., Kirschner (1980), Weisenberg (1980), Cote & Borisy (1981), Bonne & Pantaloni (1982), Carlier & Pantaloni (1982), Hill & Chen (1984), and Caplow & Reid (1985)].

GDP, on the other hand, is generally viewed as an inhibitor

[1] Abbreviations: MAPs, microtubule-associated proteins; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; p(CH_2)ppG, guanosine 5′-(β,γ-methylenetriphosphate); ATP, adenosine 5′-triphosphate; UTP, uridine 5′-triphosphate; Mes, 2-(*N*-morpholino)-ethanesulfonate; Pipes, 1,4-piperazinediethanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; EDTA, ethylenediaminetetraacetate; DEAE, diethylaminoethyl.

of tubulin polymerization (Arai & Kaziro, 1977; Carlier & Pantaloni, 1978; MacNeal & Purich, 1978; Jameson & Caplow, 1980; Zackroff et al., 1980), although its potency varies considerably with reaction conditions (Huang et al., 1985b; Duanmu et al., 1986; Hamel et al., 1986). Despite the inhibitory effects of GDP on microtubule assembly, incorporation of tubulin–GDP onto microtubule seeds (Carlier & Pantaloni, 1978; Karr et al., 1979; Zackroff et al., 1980) has been described, and a number of workers have noted at least partial stability of steady-state microtubules in GDP (Weisenberg et al., 1976; Margolis, 1981; Zeeberg & Caplow, 1981), implying that tubulin–GDP can add to microtubule ends. We are unaware, however, of any study in which the direct (i.e., without GTP hydrolysis) incorporation of exchangeable-site GDP into microtubules has been examined throughout a GTP-dependent polymerization cycle, but, as this study was nearing completion, Manser and Bayley (1985) reported direct incorporation of GDP into microtubules when assembly was induced with $p(CH_2)ppG$ and in pyrophosphate-induced polymerization of tubulin partially depleted of exchangeable nucleotide by alkaline phosphatase treatment (Bayley & Manser, 1985).

We have previously reported, in studies of glutamate- (Hamel et al., 1984) and glycerol-induced (Duanmu et al., 1986) polymerization of purified tubulin, that significant amounts of exchangeable-site GDP can be directly incorporated into polymer without being initially exchanged for exogenously added GTP. GTP was nonetheless required for polymerization to occur in both reaction systems. In glutamate this direct incorporation of GDP into polymer was observed at high tubulin concentrations, with about one-half the GDP in the polymer incorporated hydrolytically (i.e., derived from the GTP used to promote the polymerization reaction) and one-half incorporated directly. In glycerol maximum direct incorporation of GDP into polymer was observed at substoichiometric GTP concentrations, with as much as 70% of the GDP in the polymer derived directly from GDP bound at the exchangeable site.

This phenomenom merited study in greater detail, but we preferred to examine it in a polymerization system requiring MAPs. In the experiments to be presented here we have used heat-treated MAPs and electrophoretically homogeneous tubulin preparations bearing either 1.9 mol of nonradiolabeled guanine nucleotide (Hamel & Lin, 1984) or 1.7 mol of [8-^{14}C]guanine nucleotide (Duanmu et al., 1986) per mole of tubulin. In both tubulin preparations the guanine nucleotide was approximately one-half GDP and one-half GTP. In the radiolabeled preparation, only the GDP was radioactive, and we have assumed that it represents exchangeable nucleotide (see Hamel et al., 1984). Both the tubulin and MAPs preparations used here had negligible amounts of nucleoside diphosphate kinase and nonspecific phosphatase activities (Hamel & Lin, 1984), eliminating significant complications in interpretation of the data.

Materials and Methods

Materials. Electrophoretically homogeneous calf brain tubulin bearing nonradiolabeled GDP in the exchangeable site (1.9 mol of guanine nucleotide/mol of tubulin) and heat-treated MAPs were prepared as described previously (Hamel & Lin, 1984), as was tubulin with [8-^{14}C]GDP in the exchangeable site (1.7 mol of guanine nucleotide/mol of tubulin) (Duanmu et al., 1986). The protein-bound nucleotide in both tubulin preparations was approximately one-half GDP and one-half GTP. All protein preparations were chromatographed on Sephadex G-50 (superfine) to remove unbound nucleotide. All nucleotides (both radiolabeled and nonradiolabeled) were repurified by ion-exchange chromatography on DEAE-Sephadex A-25 and appeared to be homogeneous. Radiolabeled nucleotides (obtained from Moravek Biochemicals) were at least 98% radiopure as judged by thin-layer chromatography on poly(ethylenimine)–cellulose. No discrete contaminants were detected, but trace amounts of radioactivity both preceded and followed the major GDP or GTP spot [autoradiograms of these preparations are presented in Hamel et al. (1986)]. Guanine nucleotide concentrations in stock solutions (radiolabeled and nonradiolabeled) were established spectrophotometrically at 252.5 nm (millimolar extinction coefficient, 13.7) and confirmed by phosphate and ribose analysis (the three methods were within 5% agreement for all preparations).

Partial Purification of Nucleoside Diphosphate Kinase from Microtubule Protein. Beginning with 19.3 g of microtubule protein, prepared as described previously (Hamel & Lin, 1984), tubulin was separated from MAPs by Mes-induced tubulin polymerization, followed by prolonged centrifugation of the MAP-enriched supernatant (Hamel & Lin, 1984). Although a substantial amount of nucleoside diphosphate kinase activity was associated with the insoluble material (presumably membrane vesicles) (Hamel & Lin, 1984), further purification was performed on the soluble activity. This protein fraction (2.0 g, containing 212 international units of nucleoside diphosphate kinase activity) was applied to a 5 × 70 cm column of DEAE-Sephacel (from Pharmacia) as described before (Hamel & Lin, 1984). Over 80% of the enzyme activity and 20% of the protein (410 mg) were not retained by the column. Solid $(NH_4)_2SO_4$ was added to the protein solution to 60% saturation, precipitated protein was harvested by centrifugation, and additional $(NH_4)_2SO_4$ was added to 100% saturation. Protein was again harvested by centrifugation, with 80% of the enzyme activity and 120 mg of protein in the 60–100% $(NH_4)_2SO_4$ fraction. The protein pellet was dissolved in a buffer solution containing 0.1 M Mes (adjusted to pH 6.9 with NaOH) and 2 mM dithiothreitol (solution A), dialyzed against solution A, clarified by centrifugation, and applied to a preparative TSK G3000SWG high-pressure liquid chromatography column (2.15 × 60 cm), which was developed with solution A. The most active fractions contained 70% of the enzyme activity and 38 mg of protein. The enzyme was then applied to a 0.9 × 3.3 cm column of hydroxyapatite (Bio-Gel HT, from Bio-Rad), which was developed with a NaH_2PO_4 gradient from 0.01 to 0.3 M in solution A. The most active fractions were pooled, concentrated with Sephadex G-200 as described previously (Hamel & Lin, 1984), and dialyzed against solution A. They contained 40% of the activity applied to the column and 0.99 mg of protein. Overall purification was about 220-fold (from the soluble MAPs fraction), and the final preparation contained 23.4 international units/mg of protein. On the basis of a molecular weight of 17 000–18 000 for the enzyme subunit (Nickerson & Wells, 1984; Huitorel et al., 1984) and the sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern (data not presented), we estimate that the final preparation was approximately 5% pure.

Methods. All reaction mixtures contained 0.1 M Mes (adjusted to pH 6.4 with NaOH) and $MgCl_2$, tubulin, MAPs, nucleotides and other components as indicated in individual experiments.

Microtubule assembly was followed turbidimetrically (Gaskin et al., 1974) in a Gilford Model 250 recording spectrophotometer equipped with a Gilford electronic tem-

perature controller. After base lines were established at 0 °C the controller was set at 37 °C, and the temperature of the reaction mixtures rose at a rate of approximately $^1/_2$ °C/s.

Analysis of the radiolabeled nucleotide content of a microtubule pellet was performed after the indicated incubation (generally 15 min at 37 °C) by diluting the 0.20–0.25 mL-reaction mixture with 2.0 mL of an isothermic solution of 50% sucrose (Terry & Purich, 1980) containing 0.1 M Mes (pH 6.4) and 0.5 mM $MgCl_2$. The sample was then centrifuged at 40000 rpm for 30 min in a Beckman Ti 50 rotor prewarmed to the reaction temperature (generally 37 °C). The supernatant was removed by aspiration, and the microtubule pellet was washed 3 times with 2 mL of the isothermic sucrose solution. The pellet was dissolved in 0.5 mL of 8 M urea, and the radioactivity and protein content of the resulting solution were determined. Generally 0.25 mL was counted, but variable volumes were used for the protein assay depending on the protein concentrations in the original reaction mixtures. The method of Lowry et al. (1951) was used for protein determination with bovine serum albumin as standard.[2] Data are expressed as picomoles of [8-^{14}C]GDP per microgram of protein in the pellet, for whenever we have examined the nucleotide in the pellet when [8-^{14}C]GTP was used to support microtubule assembly, less than 3% GTP was found [see Hamel et al. (1986)].

Formation of [8-^{14}C]GTP from [8-^{14}C]GDP was followed by thin-layer chromatography on poly(ethylenimine)–cellulose. At desired time points 10 μL of a reaction mixture was mixed with 20 μL of 25% acetic acid containing 20 nmol each of nonradiolabeled GDP and GTP as markers; 25 μL of the mixture was spotted on a 20 × 20 cm thin-layer sheet, and chromatography was begun in 1.0 M KH_2PO_4 (pH unadjusted) while the spots were still wet. The GDP and GTP markers were visualized under ultraviolet light, cut from the thin-layer sheet, and radioactivity was determined in a liquid scintillation counter.

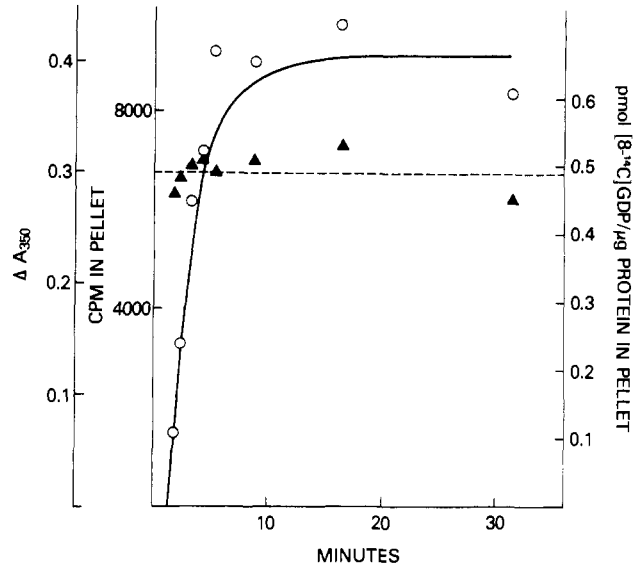

FIGURE 1: Time course for direct incorporation into microtubules of [8-^{14}C]GDP bound at the exchangeable site of tubulin. A 2.25-mL reaction mixture was prepared on ice and contained 0.1 M Mes (pH 6.4), 0.5 mM $MgCl_2$, 40 μM nonradiolabeled GTP, 0.67 mg/mL heat-treated MAPs, and 2.0 mg/mL tubulin with [8-^{14}C]GDP bound in the exchangeable site. Aliquots (0.5 mL) of the reaction mixture were placed in four cuvettes held at 0 °C by the electronic temperature controller. A 0.20-mL portion of the remainder was diluted with 2.0 mL of isothermic 50% sucrose and centrifuged at 0 °C as described in the text. After processing as described in the text, the "pellet" obtained from this control sample contained 700 cpm, and this value was subtracted from all the incubated samples. Meanwhile, the temperature controller was set at 30 °C (the reaction at 37 °C proved to be too rapid for accurate evaluation). Turbidity tracings were obtained from all four cuvettes (a minimum volume of 0.25 mL must be in the cuvettes to obtain readings), and these were essentially identical (the solid line is a composite of these curves). At the indicated times, 0.20 mL was removed from different cuvettes in succession, diluted with 2.0 mL of isothermic 50% sucrose, centrifuged, and analyzed as described in the text. Symbols: O, total cpm in pellet; ▲, ratio of radioactivity to protein in pellet, expressed as picomoles of [8-^{14}C]GDP per microgram of protein.

RESULTS

As will be discussed below, following microtubule assembly using the [8-^{14}C]GDP–tubulin preparation, substantial radioactivity is recovered in the microtubule pellet following centrifugation, depending on precise reaction conditions. Centrifugation of this tubulin, together with MAPs, in the cold or without GTP (i.e., without assembly) results in the sedimentation of smaller, but significant, amounts of protein and radioactivity, presumably representing both denatured protein and ring oligomers (the latter have been demonstrated in appropriate electron micrographs). This background could be virtually eliminated by diluting reaction mixtures with isothermic solutions of 50% sucrose prior to centrifugation (Terry & Purich, 1980). When this was done, about 3% of the original protein in a 0.25-mL reaction mixture (1 mg/mL tubulin, 0.4 mg/mL MAPs) and 300 cpm were recovered in a pellet following centrifugation (this should be contrasted to 30% of the protein and 46000 cpm in the pellet when the [8-^{14}C]GDP–tubulin and MAPs were incubated with UTP and exogenously added nucleoside diphosphate kinase[3]). Centrifugation conditions were thus chosen to minimize background radioactivity and protein in the pellet with as little loss as possible of microtubules. We do assume, moreover, in the studies presented here, that the nucleotide content of microtubules recovered in the pellet is representative of that of the entire microtubule population.

Even though dilution of the reaction mixtures with an isothermic sucrose solution was effective both in minimizing background radioactivity and in preserving adequate amounts of microtubules for analysis, we wanted additional evidence that [8-^{14}C]GDP bound in the exchangeable site was directly incorporated into microtubules. A time course study was therefore performed (Figure 1). If radioactivity in the pellet was caused by denatured protein, one would predict little change or perhaps a slow increase as the incubation proceeded; if caused by ring oligomers, the prediction would be either little change or a decrease with incubation time, depending on whether the rings were stable or dissociated and exchanged their nucleotide as the incubation proceeded. What was observed, however, was an initial rapid increase in radioactivity

[2] When known weights of lyophilized nucleotide-free tubulin and commercial bovine serum albumin, both dissolved in 8 M urea, were compared in the assay of Lowry et al. (1951), identical results were obtained in the range of 5–40 μg/mL.

[3] Centrifugation of similar reaction mixtures without dilution with the isothermic sucrose solution results in substantially larger pellets, containing about 50% of the protein in the reaction mixtures. We are uncertain at this point how the protein that does not sediment in isothermic sucrose solutions at 40000 rpm for 30 min is distributed between microtubules (presumably the shorter species), aggregated and/or denatured tubulin, and oligomeric structures.

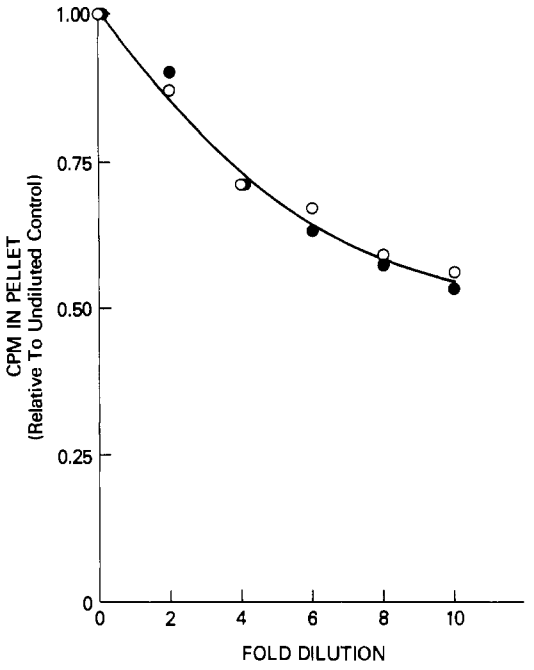

FIGURE 2: Loss of radiolabeled nucleotide from the microtubule pellet following dilutional disassembly. Two 1.3-mL reaction mixtures were prepared, each containing 1.0 mg/mL heat-treated MAPs, 0.1 M Mes (pH 6.4), and 0.5 mM $MgCl_2$. One reaction mixture also contained 60 μM nonradiolabeled GTP and 3.0 mg/mL tubulin bearing [8-^{14}C]GDP in the exchangeable site (○), while the other also contained 60 μM [8-^{14}C]GTP and 3.0 mg/mL tubulin bearing nonradiolabeled GDP in the exchangeable site (●). After 15 min at 37 °C both reaction mixtures were processed as follows (all operations at 37 °C): six 0.2-mL aliquots were placed in centrifuge tubes with different volumes of a solution containing 0.1 M Mes (pH 6.4) and 0.5 mM $MgCl_2$ (no buffer, 0.2 mL, 0.6 mL, 1.0 mL, 1.4 mL, 1.8 mL—represented by successive symbols in the figure). The samples were thoroughly mixed and then incubated at 37 °C for another 15 min. At this point 6.0 mL of a solution containing 60% sucrose, 0.1 M Mes (pH 6.4), and 0.5 mM $MgCl_2$ was added to all samples, which were thoroughly mixed, and the volume of all samples was adjusted, as necessary, to a final volume of 8.0 mL with the solution containing 0.1 M Mes (pH 6.4) and 0.5 mM $MgCl_2$. Centrifugation and analysis were performed as described in the text. (The pellets from the samples that were not diluted initially were about one-half the size of comparable pellets diluted with 2.0 mL of isothermic 50% sucrose as described in the text.) The data are expressed as cpm in the pellets relative to the samples not diluted at 15 min with the Mes–$MgCl_2$ solution (these values were 7900 cpm in the pellet obtained from the reaction mixture with tubulin bearing [8-^{14}C]GDP and 81 200 cpm in the pellet obtained from the reaction mixture with [8-^{14}C]GTP).

in the pellet, followed by an extended plateau level. The increase in total radioactivity in the pellet closely paralleled the simultaneously obtained turbidity tracing. If the ratio of radioactivity to protein is considered, one would predict a steady decline if the radioactivity in the pellet was due to either denatured protein or oligomeric structures, for as the incubation time increases steadily larger amounts of microtubules are formed and recovered in the pellet until the turbidity plateau is reached. What was observed, however, was a fairly constant ratio of radioactivity to protein in the pellet. Thus both the total radioactivity in the pellet and the ratio of radioactivity to protein are consistent with the entry of [8-^{14}C]GDP directly into microtubules.

To verify further that the directly incorporated [8-^{14}C]GDP was in microtubules and not an alternate polymer, loss of this label was compared to loss of [8-^{14}C]GDP derived from [8-^{14}C]GTP by hydrolysis (see also below) under conditions in which microtubules were partially depolymerized by dilution of the reaction mixture (Figure 2). In this experiment there was a virtually parallel loss of radioactivity from the microtubule pellet as a function of dilution, regardless of whether the radiolabeled nucleotide was initially bound to tubulin as GDP or added exogenously to the reaction mixture as GTP.[4]

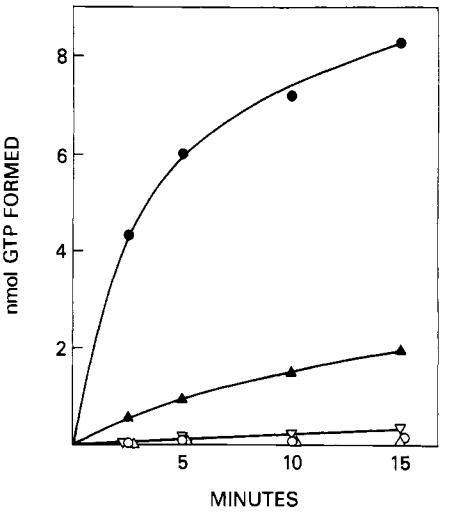

FIGURE 3: Absence of a guanosine nucleotide transphosphorylase activity in tubulin and heat-treated MAPs preparations. Each 60-μL reaction mixture contained 0.1 M Mes (pH 6.4), 0.5 mM $MgCl_2$, 2.0 mg/mL tubulin bearing [8-^{14}C]GDP in the exchangeable site, 0.67 mg/mL heat-treated MAPs, 20 μM nonradiolabeled GTP, 100 μM podophyllotoxin, and one of the following concentrations of partially purified, microtubule protein-derived nucleoside diphosphate kinase: (△) none, (●) 6 international milliunits (imu)/mL, (▲) 1.5 imu/mL, (▽) 0.3 imu/mL, or (○) 0.06 imu/mL. After the indicated times at 37 °C, 10-μL aliquots of the reaction mixtures were processed as described in the text. (Because of the potency of the nucleoside diphosphate kinase preparation, only 3.1 μg/mL enzyme was present at the highest concentration. Consequently, serial dilutions were made in bovine serum albumin solutions, and all reaction mixtures in this experiment contained 40 μg of albumin.)

Another major concern was that [8-^{14}C]GDP was incorporated into microtubules after initially being converted to GTP. Although we have found that repeated cycles of glutamate-induced polymerization eliminates nucleoside diphosphate kinase activity from tubulin (Hamel & Lin, 1981, 1984) and that heat treatment of MAPs destroys the enzyme (Hamel & Lin, 1984) as measured by phosphate transfer from ATP to GDP, it remained possible that an alternate transphosphorylase activity was present in the protein preparation(s) and was capable of transferring a phosphate group from nonradiolabeled GTP to [8-^{14}C]GDP. The study presented in Figure 3 (△) demonstrates that this is not the case. In this experiment all reaction mixtures contained 20 μM nonradiolabeled GTP, 2.0 mg/mL tubulin bearing [8-^{14}C]GDP in the exchangeable site, 0.67 mg/mL heat-treated MAPs, as well as 100 μM podophyllotoxin to prevent hydrolysis of any [8-^{14}C]GTP that might be formed [podophyllotoxin inhibits tu-

[4] The pattern of dilutional depolymerization observed in the experiment presented in Figure 2 differs from that predicted (the amount of residual polymer should be a linear function of the extent of dilution) by the nucleation–condensation model of Oosawa & Kasai (1962). Under the reaction conditions used in the experiment of Figure 2 depolymerization has been more extensive than expected at low dilutions (e.g., 2-fold) and less extensive than expected at high dilutions (e.g., 10-fold). Nevertheless, there is no evidence that residual polymer at 10-fold dilution does not consist of microtubules. Electron micrographs of negatively stained specimens following 10-fold dilution (without sucrose) and incubation as described in Figure 2 demonstrate abundant microtubules, with no other morphologically discrete structure observed aside from an occasional ring oligomer. Moreover, radioactivity in pellets from both undiluted and 10-fold diluted polymerized reaction mixtures containing either tubulin–GDP and [8-^{14}C]GTP or [8-^{14}C]GDP–tubulin and GTP returned to base line levels following depolymerization with either a 0 °C incubation or treatment with Ca^{2+}.

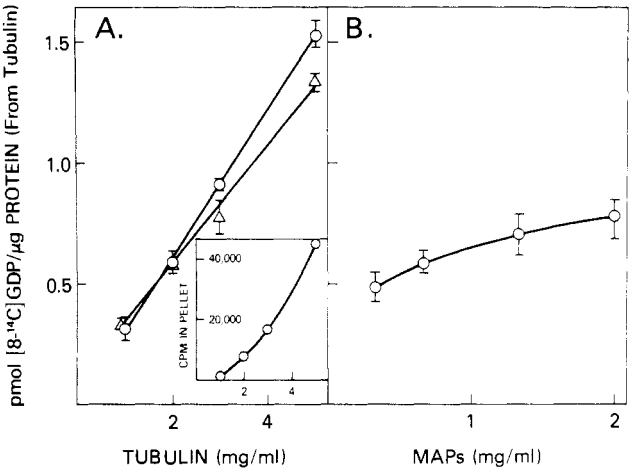

FIGURE 4: Effect of tubulin and MAPs concentrations on direct incorporation into microtubules of [8-^{14}C]GDP bound in the exchangeable site of tubulin. All 0.25-mL reaction mixtures contained 0.1 M Mes (pH 6.4), 0.5 mM $MgCl_2$, and 40 μM nonradiolabeled GTP. They were incubated at 37 °C for 15 min and diluted with isothermic 50% sucrose, centrifuged, processed, and analyzed as described in the text. The symbols in the figure represent the average of triplicate determinations, with the range indicated. (A) Effect of tubulin concentration. Reaction mixtures contained the indicated amount of tubulin with [8-^{14}C]GDP bound in the exchangeable site and either 0.67 mg/mL heat-treated MAPs (△) or heat-treated MAPs in a 1:3 weight ratio to the tubulin (○). In the main panel the data are expressed as pmol of [8-^{14}C]GDP/μg of protein in the pellet. In the inset, the total cpm in the pellets (average of the triplicates) of the second series (MAPs:tubulin = 1:3) is presented. (B) Effect of MAPs concentration. Reaction mixtures contained 2.0 mg/mL tubulin with [8-^{14}C]GDP in the exchangeable site and the indicated concentrations of heat-treated MAPs. Data are expressed as picomole of [8-^{14}C]GDP per microgram of protein in the pellet.

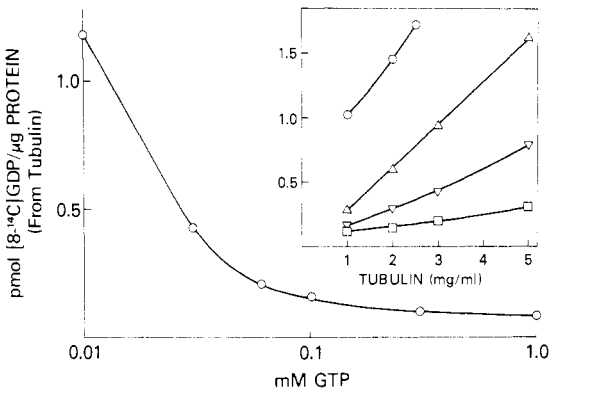

FIGURE 5: Effect of reaction GTP concentration on direct incorporation into microtubules of [8-^{14}C]GDP bound in the exchangeable site of tubulin. Each 0.25-mL reaction mixture contained 1.0 mg/mL tubulin with [8-^{14}C]GDP bound in the exchangeable site, 0.40 mg/mL heat-treated MAPs, 0.1 M Mes (pH 6.4), 0.5 mM $MgCl_2$, and the indicated concentration of nonradiolabeled GTP. After 15 min at 37 °C reaction mixtures were diluted with isothermic 50% sucrose, centrifuged, processed, and analyzed as described in the text. (Inset) Interrelationship of tubulin and GTP concentrations in the amount of direct incorporation into microtubules of [8-^{14}C]GDP bound in the exchangeable site of tubulin. Reaction conditions and sample processing and analysis were as described for the main panel, except that reaction mixtures contained the indicated amounts of tubulin with [8-^{14}C]GDP bound in the exchangeable site, heat-treated MAPs in a 1:3 weight ratio to the tubulin, and nonradiolabeled GTP as follows: (○) 10 μM, (△) 40 μM, (▽) 100 μM, (□) 1000 μM.

bulin-dependent GTP hydrolysis (David-Pfeuty et al., 1979) but does not affect nucleotide exchange on tubulin (Huang et al., 1985a)].

To verify that any [8-^{14}C]GTP formed would have been detected, as well as to estimate an upper limit for the amount of residual nucleoside diphosphate kinase that might remain in the protein preparation(s), serial dilutions of a nucleoside diphosphate kinase preparation partially purified from microtubule protein were added to identical reaction mixtures containing tubulin bearing [8-^{14}C]GDP in the exchangeable site, heat-treated MAPs, nonradiolabeled GTP, and podophyllotoxin (Figure 3). With 6 international milliunits/mL enzyme (equivalent to approximately 10% of the nucleoside diphosphate kinase that would be associated with 2.0 mg/mL tubulin in microtubule protein), rapid formation of GTP occurred: by 15 min almost one-half of the [8-^{14}C]GDP initially bound to tubulin had been transphosphorylated. The reactions with serial dilutions of the enzyme indicated that less than 0.3 international milliunit/mL nucleoside diphosphate kinase is present in reaction mixtures containing 2.0 mg/mL tubulin and 0.67 mg/mL heat-treated MAPs.[5]

In preparing the tubulin bearing [8-^{14}C]GDP in the exchangeable site, we had used cycles of glutamate-induced polymerization with the tubulin at 40–50 mg/mL and [8-^{14}C]GTP at 1 mM (tubulin:GTP ≈ 1:2) (Hamel et al., 1984; Duanmu et al., 1986). Under this condition we have repeatedly observed that only 50% of the tubulin in the polymer bound radiolabeled GDP following polymerization, polymer isolation, and depolymerization (i.e., the GDP isolated from the tubulin had only one-half the specific activity of the [8-^{14}C]GTP used to support polymerization, while the tubulin-bound GTP was completely nonradioactive and presumably derived from the nonexchangeable site). Incorporation of radiolabeled nucleotide was enhanced by either repetitive polymerization cycles or lowering the tubulin concentration to 5 mg/mL. This latter observation led us to first examine the effects of protein concentration on the direct incorporation of [8-^{14}C]GDP into microtubules under conditions in which the tubulin to GTP ratio was relatively high (ranging from 1:4 to 5:4). Figure 4A contrasts a study in which the tubulin to MAPs weight ratio was held constant at 3:1 (circles) to an experiment in which the MAPs were invariant (triangles). Under both conditions there was a steady, almost linear, rise in the ratio of radiolabeled GDP to protein recovered in the microtubule pellet as the tubulin concentration rose from 1.0 to 5.0 mg/mL. Further, there were only small differences in the ratio of radioactivity to protein whether the amount of MAPs was held constant or increased with the tubulin (total counts and protein recovered were, however, significantly higher when both MAPs and tubulin were increased as compared to increasing tubulin only with the MAPs held constant). It should be noted that while the nucleotide to protein ratio was a linear function of tubulin concentration (from 1 to 5 mg/mL), total counts in the pellet rose exponentially (Figure 4A, inset), as should occur with increased formation of microtubules in combination with a rising radioactivity to protein ratio. Altering the MAPs to tubulin ratio by increasing the MAPs, on the other hand, had only minor effects on the ratio of radioactivity to protein in the microtubule pellet (Figure 4B).

One would predict that the direct incorporation of [8-^{14}C]GDP into the microtubule pellet would be affected by the

[5] Centrifugation of similar reaction mixtures (without podophyllotoxin) resulted in a pellet containing 1.3 pmol of [8-^{14}C]GDP/μg of protein if no nucleoside diphosphate kinase was added, 1.3 pmol/μg with 0.06 international milliunit (imu)/mL enzyme, 1.4 pmol/μg with 0.3 imu/mL, 1.7 pmol/μg with 1.5 imu/mL, and 2.1 pmol/μg with 6.0 imu/mL enzyme. Thus, although trace amounts of nucleoside diphosphate kinase do not affect the amount of [8-^{14}C]GDP incorporated directly into microtubules, the amount of radiolabeled GDP that enters the pellet increases with larger amounts of enzyme as would be predicted.

Table I: Effects of Reaction Conditions on Direct Incorporation of GDP into Microtubules[a]

reaction condition	pmol of [8-^{14}C]GDP/μg of protein[b]
standard[c]	0.8 (0.77–0.82)
pH 6.0	0.7 (0.65–0.73)
pH 7.0	0.8 (0.78–0.93)
0.1 M Pipes (pH 7.0)[d]	0.8 (0.72–0.94)
25 °C	0.9 (0.89–0.90)
30 °C	0.9 (0.88–0.90)
+1 mM EGTA	0.8 (0.81–0.88)
+2 mM $MgCl_2$[e]	0.6 (0.58–0.63)
+5 mM $MgCl_2$[e]	0.5 (0.40–0.50)
$-MgCl_2$	1.9 (1.83–1.92)
$-MgCl_2$, +0.2 mM EDTA	2.3 (2.28–2.47)

[a] Incorporation of [8-^{14}C]GDP originally bound in the exchangeable site into a microtubule pellet was determined as described in detail in the text. [b] The average value obtained in three independent experiments is presented, with the range of values obtained presented in parentheses. [c] Standard condition was a 0.25-mL reaction mixture containing 2.0 mg/mL tubulin with [8-^{14}C]GDP in the exchangeable site, 0.67 mg/mL heat-treated MAPs, 30 μM GTP, 0.1 M Mes adjusted to pH 6.4 with NaOH, and 0.5 mM $MgCl_2$. Incubation was at 37 °C for 15 min prior to dilution with a 50% isothermic sucrose solution containing 0.1 M Mes (pH 6.4) and 0.5 mM $MgCl_2$. Modifications of this standard condition are indicated in the table. [d] NaOH was used to adjust the pH. [e] Final concentration.

concentration of exogenously added GTP in the reaction mixture. This is clearly the case. The experiment presented in the main panel of Figure 5 documents the rapid fall in [8-^{14}C]GDP recovered in the microtubule pellet as the concentration of exogenously added GTP was raised from 10 μM (stoichiometric with the tubulin at 1 mg/mL) to 1 mM, with 90% of the drop occurring by 60–100 μM GTP. The inset presents a more extensive experiment in which the interrelationship of tubulin and GTP concentrations was examined. At every GTP concentration, more [8-^{14}C]GDP was directly incorporated into microtubules at higher tubulin concentrations, and conversely, at every tubulin concentration direct incorporation of [8-^{14}C]GDP was greater the lower the GTP concentration.

A variety of reaction conditions were next examined for potential effects on direct incorporation of [8-^{14}C]GDP into microtubules (Table I). Reaction pH, substitution of Pipes for Mes, reaction temperature, and addition of EGTA to the reaction mixture had minimal, probably insignificant, effects on the ratio of radioactivity to protein in the pellet. Changes in the Mg^{2+} concentration, on the other hand, caused significant alterations in this ratio. Increasing the Mg^{2+} concentration resulted in a small decrease in the ratio of radioactivity to protein in the microtubule pellet, while eliminating exogenous Mg^{2+} caused the ratio to more than double. Addition of EDTA to the reaction mixture resulted in a further increase in direct incorporation of radiolabeled GDP into the microtubule pellet.

A prediction derived from these observations on the direct incorporation of GDP into microtubules is that at lower GTP and higher tubulin concentrations the proportion of GDP derived from GTP in polymerization should be reduced. This has been confirmed in a number of studies. In that presented in Figure 6, tubulin (2.5 mg/mL) bearing either [8-^{14}C]GDP or nonradiolabeled GDP was polymerized with, respectively, nonradiolabeled GTP or [8-^{14}C]GTP. [In experiments presented elsewhere we have demonstrated that virtually all the radiolabeled nucleotide in the microtubules following polymerization with radiolabeled GTP is in the form of GDP (Hamel et al., 1986)—see also Caplow & Zeeberg (1980).] At the

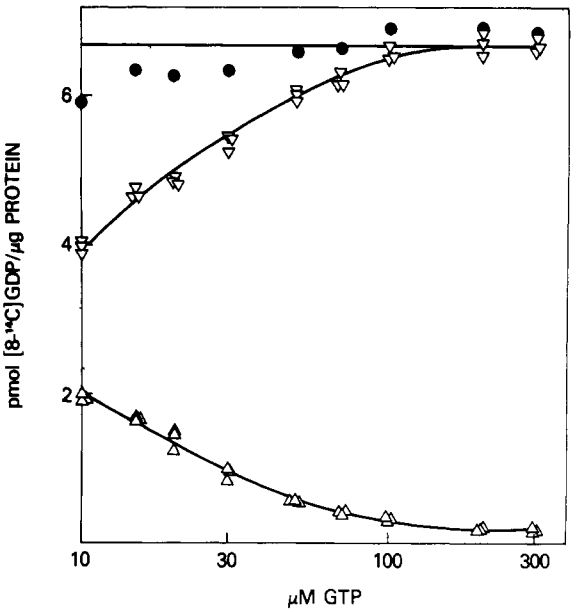

FIGURE 6: Effect of reaction GTP concentration on relative amounts of GDP in microtubules derived from GTP (hydrolytic incorporation) and from GDP initially bound in the exchangeable site (direct incorporation). The experiment was performed 3 times, and the range of values obtained is presented in the figure. Samples were incubated for 15 min at 37 °C and diluted with isothermic 50% sucrose, centrifuged, processed, and analyzed as described in the text. The symbols △ represent 0.25-mL reaction mixtures containing 2.5 mg/mL tubulin with [8-^{14}C]GDP bound in the exchangeable site, 0.83 mg/mL heat-treated MAPs, 0.1 M Mes (pH 6.4), 0.2 mM $MgCl_2$, and the indicated concentrations of nonradiolabeled GTP. The symbols ▽ represent 0.25-mL reaction mixtures containing 2.5 mg/mL tubulin with nonradiolabeled GDP bound in the exchangeable site, 0.83 mg/mL heat-treated MAPs, 0.1 M Mes (pH 6.4), 0.2 mM $MgCl_2$, and the indicated concentrations of [8-^{14}C]GTP. The symbols ● represent the total amount of GDP in microtubules at each GTP concentration (sum of the average values obtained under the two different conditions).

lower GTP concentrations, there was a significant reduction in the polymer [8-^{14}C]GDP derived from GTP. This almost exactly compensated for the increased polymer [8-^{14}C]GDP derived from tubulin, for across the entire GTP concentration range studied the total [8-^{14}C]GDP in microtubules varied minimally. [Analysis of reaction mixtures and microtubule pellets by polyacrylamide gel electrophoresis demonstrated that tubulin and MAPs were in the same proportions in the pellets as in the reaction mixtures. Thus the 6.52 pmol of GDP/μg of protein (average of all the sums in Figure 6) represents 0.87 pmol of GDP/pmol of tubulin, or 87% saturation.]

The observation of direct incorporation of GDP into microtubules raised the question of whether GDP initially bound in the exchangeable site differs from exogenously added GDP—i.e., whether the latter will also enter microtubules despite its inhibitory effect on polymerization (Figure 7, inset). In the experiment presented in the main panel of Figure 7 tubulin was incubated with a constant amount of GTP and increasing amounts of GDP in one of these combinations: (1) [8-^{14}C]GDP in the exchangeable site and nonradiolabeled exogenous nucleotides or (2) and (3) nonradiolabeled GDP in the exchangeable site with either the exogenous GDP or the exogenous GTP labeled in the 8-position with ^{14}C. (These studies were limited to exogenous GDP concentrations, ≤60 μM, that had minimal effects on the final turbidity plateau—see Figure 7, inset.) Increasing the concentration of GDP in the reaction mixture resulted in a steady rise in the proportion of exogenously added GDP to protein in the microtubule pellet, together with a fall in direct incorporation

of [8-^{14}C]GDP and a still greater drop in the amount of nucleotide derived from the exogenously added [8-^{14}C]GTP. The proportion of total nucleotide to protein again was essentially constant (the average of all the sums in Figure 7 was 6.36 pmol/μg protein, nearly identical with that obtained in the study of Figure 6). It should also be noted that the relative amounts of directly incorporated GDP and exogenous GDP in the microtubule pellets were similar to the relative proportions of tubulin and GDP in the reaction mixtures (i.e., with GDP:tubulin = 0.6, 0.6 pmol of GDP/μg of protein was derived from exogenous GDP, while 0.8 pmol of GDP/μg of protein was directly incorporated, but with GDP:tubulin = 1.8, 1.4 pmol of GDP/μg of protein was derived from exogenous GDP, while 0.6 pmol of GDP/μg of protein was directly incorporated). This result is consistent with complete exchangeability of the tubulin-bound GDP under the reaction conditions used here,[6] and indicates no significant difference between the two potential pools of GDP that can enter microtubules during assembly.

The GDP in microtubules can thus be derived from several sources. Most of it will generally come from GTP hydrolyzed to GDP during polymerization; but, depending on precise reaction conditions, variable amounts will come from GDP previously in polymer that remains bound to tubulin following a depolymerization step or from GDP that has entered the exchangeable site after depolymerization. In all cases the microtubule nucleotide content is nevertheless identical, consisting of GDP.

Discussion

In the studies presented here we have unambiguously documented that GDP in the exchangeable site of tubulin can be directly incorporated into microtubules under typical reaction conditions in which both GTP and MAPs are required for polymerization. Thus, a significant proportion of tubulin dimers (either as dimers or as components of oligomers—see below) can enter microtubules without an initial GDP–GTP exchange reaction and subsequent GTP hydrolysis. The exact proportion depends on specific reaction conditions. Direct incorporation of GDP into microtubules is favored by a high tubulin concentration, a low GTP concentration, a low Mg^{2+} concentration, and free GDP in the reaction mixture. Hydrolytic incorporation of GDP into microtubules (i.e., GDP derived from GTP by hydrolysis during the current polymerization cycle) is favored by the opposite conditions—low tubulin, high GTP or Mg^{2+}, and minimal free GDP.

Other workers have previously demonstrated an increase in turbidity when microtubules or microtubule seeds were added to solutions of tubulin containing predominantly GDP in the exchangeable site (Carlier & Pantaloni, 1978; Karr et al., 1979; Zackroff et al., 1980). These findings implied the direct addition of tubulin–GDP onto microtubules and that GTP hydrolysis was not essential for microtubule elongation. More recently, Bayley's laboratory has reported direct incorporation of GDP into microtubules under two unusual reaction conditions: polymerization dependent on p(CH_2)ppG (Manser & Bayley, 1985) and pyrophosphate-dependent polymerization of tubulin partially depleted of exchangeable nucleotide by alkaline phosphatase treatment (Bayley & Manser, 1985). The studies presented here specifically demonstrate that direct incorporation (as well as hydrolytic incorporation) of GDP into microtubules occurs throughout an assembly cycle (Figure 1), with its extent determined by specific reaction conditions. It is important to note, moreover, that our findings demonstrate that under virtually all reaction conditions some tubulin–GDP will enter microtubules without nucleotide exchange and hydrolysis.

The effects of tubulin, GDP, and GTP concentrations on direct incorporation of GDP into microtubules are qualitatively in accord with what would be predicted as the effects of these agents on the ratio of GDP and GTP bound at the exchangeable site of tubulin prior to the onset of assembly. It

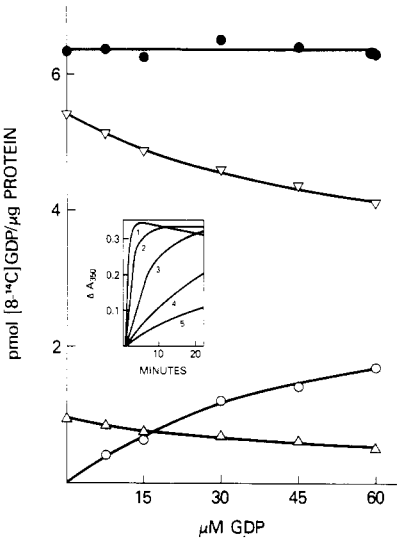

FIGURE 7: Effect of exogenous GDP on relative amounts of GDP in microtubules derived from GTP, from exogenously added GDP, and from GDP initially bound in the exchangeable site. The experiment was performed 3 times, but only the average values obtained are presented in the figure (the range of values obtained was very similar to that presented in Figure 6). Samples were incubated for 15 min at 37 °C and diluted with isothermic 50% sucrose, centrifuged, processed, and analyzed as described in the text. The symbols Δ represent 0.25-mL reaction mixtures containing 2.5 mg/mL tubulin with [8-^{14}C]GDP bound in the exchangeable site, 0.83 mg/mL heat-treated MAPs, 30 μM nonradiolabeled GTP, 0.1 M Mes (pH 6.4), 0.2 mM $MgCl_2$, and the indicated amounts of nonradiolabeled GDP. The symbols ∇ represent 0.25-mL reaction mixtures containing 2.5 mg/mL tubulin with nonradiolabeled GDP in the exchangeable site, 0.83 mg/mL heat-treated MAPs, 30 μM [8-^{14}C]GTP, 0.1 M Mes (pH 6.4), 0.2 mM $MgCl_2$, and the indicated amounts of nonradiolabeled GDP. The symbols ○ represent 0.25-mL reaction mixtures containing 2.5 mg/mL tubulin with nonradiolabeled GDP in the exchangeable site, 0.83 mg/mL heat-treated MAPs, 30 μM nonradiolabeled GTP, 0.1 M Mes (pH 6.4), 0.2 mM $MgCl_2$, and the indicated amounts of [8-^{14}C]GDP. The symbols ● represent the total amount of GDP in microtubules at each GDP concentration (sum of the average values obtained under the three different conditions). (Inset) Inhibitory effects on microtubule assembly of increasing GDP concentration under the reaction conditions used in the main panel. The temperature controller was set at 37 °C at zero time. Curve 1, no GDP; curve 2, 30 μM GDP; curve 3, 60 μM GDP; curve 4, 90 μM GDP; curve 5, 120 μM GDP.

[6] At first glance, this seems to indicate a somewhat excessive incorporation of exogenous GDP into the microtubule pellet. However, the [8-^{14}C]GDP–tubulin was initially only 85% saturated with nucleotide, while the nonradiolabeled tubulin was 95% saturated with nucleotide. Thus, with 2.5 mg/mL tubulin, the reaction mixtures with radiolabeled endogenous GDP initially contained the equivalent of 21 μM [8-^{14}C]GDP while those with nonradiolabeled endogenous GDP initially contained the equivalent of 24 μM nonradiolabeled GDP. Including these values and assuming complete GDP exchange at 0 °C, prior to the onset of polymerization, the calculated values for radiolabeled GDP in the microtubule pellet are as follows: at 15 μM exogenous GDP, 0.82 pmol of [8-^{14}C]-GDP/μg of protein should be derived from endogenous [8-^{14}C]GDP and 0.54 pmol from exogenous [8-^{14}C]GDP (the experimental values were 0.75 and 0.64, respectively); at 45 μM exogenous GDP, 0.65 pmol of [8-^{14}C]GDP should be derived from endogenous [8-^{14}C]GDP and 1.33 pmol from exogenous [8-^{14}C]GDP (the experimental values were 0.62 and 1.42, respectively). There was similar reasonable agreement between calculated and experimental values at the three additional GDP concentrations examined in the study of Figure 7.

thus seems likely that the precise amount of direct incorporation of GDP into microtubules must bear a direct relationship to the equilibrium of GDP and GTP at the exchangeable site under differing reaction conditions. We are at present attempting to define this relationship quantitatively. In addition, the effect of Mg^{2+} on direct incorporation of GDP into microtubules is consistent with the effect of the cation on the binding of GDP and GTP to tubulin; Mg^{2+} is not required for the binding of GDP to tubulin but is essential for the optimal binding of GTP to the protein (Huang et al., 1985b).

Practically speaking, since most in vitro studies of microtubule assembly are performed at relatively high GTP concentrations in the presence of Mg^{2+}, there are at least four circumstances when significant amounts of direct incorporation of GDP might occur and should be considered in a complete description of microtubule assembly: (1) at high tubulin concentrations, (2) at GDP concentrations that partially inhibit polymerization, (3) when GDP derived from GTP used in the preparation and/or stabilization of tubulin is added in undefined amounts along with tubulin to the reaction mixture, and (4) when GDP is generated from GTP by phosphatase activities present in microtubule protein preparations.

Since nominally equivalent microtubules can in fact be formed in different reaction pathways, an equation such as

$$\text{tubulin–GTP} \rightarrow \text{microtubule–GDP} \rightleftarrows \text{tubulin–GDP}$$

is inadequate to describe microtubule assembly in which different amounts of direct and hydrolytic GDP incorporation occurs. A more appropriate scheme must include an indication that these two mechanisms of tubulin incorporation into microtubules can occur and may affect the polymer differently:[7]

$$\text{tubulin–GTP} \rightarrow \text{microtubule}_{\text{H}}\text{–GDP} \rightarrow \text{tubulin–GDP} \rightleftarrows \text{microtubule}_{\text{D}}\text{–GDP}$$

Or some somewhat more accurately, since the experiments presented here demonstrate that microtubules can be varying composites of "microtubule$_H$" and "microtubule$_D$" units

$$m(\text{tubulin–GTP}) + n(\text{tubulin–GDP}) \rightarrow (\text{microtubule}_{\text{H}}\text{–GDP})_m\text{–}(\text{microtubule}_{\text{D}}\text{–GDP})_n \rightarrow (m + n)(\text{tubulin–GDP})$$

While the relative values of m and n are dependent on the specific GTP and GDP concentrations and the K_D values for these nucleotides with tubulin under specific reaction conditions, what is uncertain at this time is whether microtubule elongation with tubulin–GDP and tubulin–GTP occurs in proportion to the relative amounts of these species in the reaction mixture or whether tubulin–GTP is more efficiently incorporated. It does appear, however, that direct and hydrolytic incorporation of GDP into microtubules occurs in a relatively constant ratio throughout an elongation cycle, within the time periods we have examined (Figure 1).

Many workers have suggested that oligomeric species consisting of tubulin + MAPs participate in microtubule assembly in the nucleation and/or elongation phases [e.g., Borisy & Olmsted (1972), Erickson (1974), Kirschner et al. (1974), Mandelkow et al. (1980), Weisenberg (1980), Barton & Riazi (1982), Burns & Islam (1984), and Bayley et al. (1985)], and in their reports of direct incorporation of GDP into microtubules in p(CH_2)ppG-dependent (Manser & Bayley, 1985) and pyrophosphate-dependent (Bayley & Manser, 1985) polymerization, Bayley and Manser speculated that the GDP was carried into the polymer by oligomeric species. Although the experiments presented here do not address the question of whether either direct or hydrolytic GDP incorporation into microtubules occurs via tubulin dimer and/or oligomer addition to the polymer, several characteristics of our system reconstituted from tubulin and heat-treated MAPs should be noted: (1) We have not yet observed discrete oligomers in the purified tubulin, but ring oligomers form when the tubulin and MAPs are mixed at 0 °C ± GTP and persist at 37 °C in the absence of GTP. (2) Unlike the minimal binding of exogenous nucleotide to the exchangeable site of tubulin in ring oligomers in porcine microtubule protein reported by Caplow and Zeeberg (1980), the exchangeable site in the ring oligomers formed in our reconstituted bovine system seems fully accessible to exogenous nucleotide. The heat-treated MAPs do not inhibit the binding of either GDP or GTP to tubulin nor prevent displacement of radiolabeled GDP from tubulin by exogenous nucleotide.[8] (3) Under appropriate reaction conditions (i.e., low tubulin and high GTP—Figure 5), direct GDP incorporation in the tubulin + MAPs system is negligible. (4) The effects of tubulin and GTP concentrations on direct GDP incorporation into microtubules with MAPs are qualitatively similar to their effects in the polymerization of purified tubulin (Hamel et al., 1984; Duanmu et al., 1986). (5) Direct incorporation of GDP into microtubules occurs to approximately the same extent both at the onset of assembly and as the reaction approaches steady state (Figure 1).

In conclusion, if oligomeric species participated in the direct incorporation of GDP into microtubules in the experiments described here, the accessibility of the exchangeable site in tubulin molecules in the oligomers was equivalent to its accessibility in dimers (rapid and complete equilibration of exogenous and endogenous nucleotide). Participation of oligomers, especially of mixed nucleotide composition, in microtubule assembly obviously would complicate the scheme outlined above, but it would not alter the need to include both hydrolytic and direct GDP incorporation in a complete description of microtubule assembly.

Registry No. GDP, 146-91-8; GTP, 86-01-1.

References

Arai, T., & Kaziro, Y. (1977) *J. Biochem. (Tokyo) 82*, 1063–1071.

Barton, J. S., & Riazi, G. H. (1982) *Biochim. Biophys. Acta 705*, 8–11.

Bayley, P. M., & Manser, E. J. (1985) *Nature (London) 318*, 683–685.

Bayley, P. M., Butler, F. M. M., Clark, D. C., Manser, E. J., & Martin, S. R. (1985) *Biochem. J. 227*, 439–455.

Bonne, D., & Pantaloni, D. (1982) *Biochemistry 21*, 1075–1081.

Borisy, G. G., & Olmsted, J. B. (1972) *Science (Washington, D.C.) 177*, 1196–1197.

Burns, R. G., & Islam, K. (1984) *FEBS Lett. 173*, 67–74.

Caplow, M., & Zeeberg, B. (1980) *Arch. Biochem. Biophys. 203*, 404–411.

[7] In preliminary experiments we have found a striking apparent inverse correlation between the amount of direct incorporation of GDP into microtubules and polymer stability to both low temperature and Ca^{2+} as a function of GTP, GDP, and tubulin concentrations. All conditions that favor more unstable polymers (high tubulin, low GTP, exogenous GDP), however, are also associated with an increased free GDP to GTP ratio in the reaction mixture as well as increased direct GDP incorporation into microtubules. We are presently attempting to determine whether it is the mechanism of microtubule assembly (direct vs. hydrolytic GDP incorporation) or the microtubule's environment (free GDP to free GTP ratio) that is the more important factor in its stability.

[8] Preliminary experiments with MAPs that had not been heat treated have yielded similar results.

Caplow, M., & Reid, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3267–3271.
Carlier, M.-F., & Pantaloni, D. (1978) *Biochemistry 17*, 1908–1915.
Carlier, M.-F., & Pantaloni, D. (1982) *Biochemistry 21*, 1215–1224.
Cote, R. H., & Borisy, G. G. (1981) *J. Mol. Biol. 150*, 577–602.
David-Pfeuty, T., Simon, C., & Pantaloni, D. (1979) *J. Biol. Chem. 254*, 11696–11702.
Duanmu, C., Lin, C. M., & Hamel, E. (1986) *Biochim. Biophys. Acta 881*, 113–123.
Erickson, H. P. (1974) *J. Supramol. Struct. 2*, 393–411.
Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737–758.
Hamel, E., & Lin, C. M. (1981) *Arch. Biochem. Biphys. 209*, 29–40.
Hamel, E., & Lin, C. M. (1984) *Biochemistry 23*, 4173–4184.
Hamel, E., Lustbader, J., & Lin, C. M. (1984) *Biochemistry 23*, 5314–5325.
Hamel, E., Batra, J. K., Huang, A. B., & Lin, C. M. (1986) *Arch. Biochem. Biophys. 245*, 316–330.
Hill, T. L., & Chen, Y.-D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5772–5776.
Huang, A. B., Lin, C. M., & Hamel, E. (1985a) *Biochem. Biophys. Res. Commun. 128*, 1239–1246.
Huang, A. B., Lin, C. M., & Hamel, E. (1985b) *Biochim. Biophys. Acta 832*, 22–32.
Huitorel, P., Simon, C., & Pantaloni, D. (1984) *Eur. J. Biochem. 144*, 233–241.
Jameson, L., & Caplow, M. (1980) *J. Biol. Chem. 255*, 2284–2292.
Karr, T. L., Podrasky, A. E., & Purich, D. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5475–5479.
Kirschner, M. W. (1980) *J. Cell Biol. 86*, 330–334.
Kirschner, M. W., Williams, R. C., Weingarten, M., & Gerhart, J. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1159–1163.
Kobayashi, T. (1975) *J. Biochem. (Tokyo) 76*, 201–204.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
MacNeal, R. K., & Purich, D. L. (1978) *J. Biol. Chem. 253*, 4683–4687.
Mandelkow, E. M., Harmsen, A., Mandelkow, E., & Bordas, J. (1980) *Nature (London) 287*, 595–599.
Manser, E. J., & Bayley, P. M. (1985) *Biochem. Biophys. Res. Commun. 131*, 386–394.
Margolis, R. L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1586–1590.
Nickerson, J. A., & Wells, W. W. (1984) *J. Biol. Chem. 259*, 11297–11304.
Oosawa, F., & Kasai, M. (1962) *J. Mol. Biol. 4*, 10–21.
Terry, B. J., & Purich, D. (1980) *J. Biol. Chem. 255*, 10532–10536.
Weisenberg, R. C. (1980) *J. Mol. Biol. 139*, 660–677.
Weisenberg, R. C., Borisy, G. G., & Taylor, E. W. (1968) *Biochemistry 7*, 4466–4477.
Weisenberg, R. C., Deery, W. J., & Dickinson, P. J. (1976) *Biochemistry 15*, 4248–4254.
Zackroff, R. V., Weisenberg, R. C., & Deery, W. J. (1980) *J. Mol. Biol. 139*, 641–659.
Zeeberg, B., & Caplow, M. (1981) *J. Biol. Chem. 256*, 12051–12057.

Chromatin Structure of the Cytochrome P-450c Gene Changes following Induction

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Received March 18, 1986; Revised Manuscript Received June 2, 1986

ABSTRACT: The chromatin structure of cytochrome P-450c and P-450d genes, which in the liver are highly inducible by 3-methylcholanthrene, was studied in normal and carcinogen-treated rats by using a cDNA probe specific for P-450c and a genomic probe that recognizes both genes. Digestion with micrococcal nuclease revealed that the active genes are not present in the typical 200 base pair nucleosomal structure. Gene induction is associated with a rearrangement of the nuclear organization of the genes. By use of indirect end-label hybridization, three DNase I hypersensitive sites were mapped, one in the 5′-terminal region and two in the 3′ region of the P-450c gene. Gene induction, by treatment with 3-methylcholanthrene, changes the location of the DNase I site present in the 5′ region without affecting the sites present in the 3′ region. Rat thymus chromatin does not contain these DNase I hypersensitive sites, suggesting that, in the liver, the chromatin structure is altered so as to allow tissue-specific expression of the P-450c gene. The chromatin structure of the highly inducible P-450c gene is compared to that of the P-450m gene, which is induced to a significantly smaller extent and is constitutively expressed.

The cytochrome P-450 family of proteins is involved in the metabolism of a variety of xenobiotic and endogenous compounds including drugs, carcinogens, and toxins (Conney, 1982; Boobis et al., 1985). Some of the metabolites produced are potent mutagens and carcinogens that bind covalently to cellular macromolecules such as proteins, RNA, and DNA (Gelboin, 1980). Synthesis of the various members of the cytochrome P-450 enzyme system are highly inducible in a very selective manner, and the isoenzymes generated have distinct yet overlapping substrate specificities (Lu & West, 1980; Adesnik & Atchison, 1985). The rapid, temporal induction of a specific set of genes in response to exposure to specific inducers is an important aspect of both the detoxification process and the production of harmful intermediates. The regulation of gene expression is therefore central to the coordinate control of this family of genes and to their biological role. Compared to total nuclear DNA, active or potentially active genes have an altered chromatin structure that can be