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Microtubule Assembly with the Guanosine 5′-Diphosphate Analogue 2′,3′-Dideoxyguanosine 5′-Diphosphate†

Ernest Hamel,* Anthony A. del Campo, and Chii M. Lin

ABSTRACT: The GDP analogue 2′,3′-dideoxyguanosine 5′-diphosphate (ddGDP) supports efficient tubulin polymerization. Microtubule-associated protein (MAP) dependent microtubule assembly occurs in 0.1 M 2-(*N*-morpholino)-ethanesulfonate, and sheets of parallel protofilaments are formed in 1.0 M glutamate without MAPs. The nucleotide is bound to tubulin in the course of polymerization, presumably in the exchangeable GTP site. The ddGDP is not hydrolyzed, however, and is completely stable in the reaction mixture. Nor was the nonexchangeable GTP bound to tubulin hydrolyzed in ddGDP-supported polymerization: equivalent amounts of GTP remained associated with polymerized tubulin after polymerization with either ddGDP or GTP. Higher concentrations of ddGDP than GTP were required under all conditions. Nevertheless, under optimum conditions for the ddGDP-supported reaction, polymerization began with a shorter lag period and both the rate and extent of polymerization were greater with ddGDP than with GTP. The MAP-dependent reaction with ddGDP is temperature dependent, cold reversible, and inhibited by calcium and antimitotic drugs. It differs from the GTP-supported reaction in being most vigorous at minimal Mg^{2+} concentrations and exquisitely sensitive to GDP inhibition.

Two guanosine nucleotide binding sites are present on tubulin (Weisenberg et al., 1968; Bryan, 1972; Kobayashi, 1974; Hamel & Lin, 1981a), the major protein component of microtubules. One of these is termed the nonexchangeable site, since the GTP which fills it cannot be removed from tubulin without denaturing the protein. The other is termed the exchangeable site, since nucleotide bound in it can be displaced by exogenous GDP or GTP. Although nonhydrolyzable GTP analogues can support tubulin polymerization (Weisenberg et al., 1976; Arai & Kaziro, 1976; Sutherland, 1976; Penningroth & Kirschner, 1977), microtubule assembly generally requires the hydrolysis of exchangeably bound GTP to GDP (Kobayashi, 1975; Weisenberg et al., 1976; Penningroth & Kirschner, 1977; Arai & Kaziro, 1977; David-Pfeuty et al., 1977; MacNeal & Purich, 1978). GDP, particularly if added prior to the onset of polymerization, inhibits the reaction (Arai & Kaziro, 1977; Carlier & Pantaloni, 1978; MacNeal & Purich, 1978; Zackroff et al., 1980; Jameson & Caplow, 1980; Hamel & Lin, 1981a).

We have been examining interactions of ribose-modified analogues of GDP and GTP at the exchangeable site to define structural requirements for the nucleotide in greater detail (Hamel & Lin, 1981b; Lustbader & Hamel, 1982; Hamel et al., 1983). One notable finding has been that while several GTP analogues were more efficient than GTP itself in supporting polymerization, no GDP analogue was as effective an inhibitor as GDP. Perhaps most striking was the contrast between ddGTP[1] and ddGDP.

† From the Laboratory of Medicinal Chemistry and Pharmacology (E.H. and C.M.L.) and the Laboratory of Experimental Therapeutics and Metabolism (A.A.d.C.), Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. *Received January 12, 1983.*

[1] Abbreviations: ddGMP, ddGDP, and ddGTP, 2′,3′-dideoxyguanosine 5′-mono-, 5′-di-, and 5′-triphosphates; MAPs, microtubule-associated proteins; Mes, 2-(*N*-morpholino)ethanesulfonate; p(CH_2)pG, guanosine 5′-(α,β-methylenediphosphate).

Polymerization with ddGTP began earlier and, in the presence of MAPs, was substantially more extensive than with GTP. Moreover, with ddGTP alone of all the nucleotides we have examined, MAPs were not required for polymerization in 0.1 M Mes–0.5 mM $MgCl_2$ (Hamel et al., 1983).

In contrast, ddGDP has virtually no inhibitory effect on triphosphate-supported polymerization (Hamel & Lin, 1981b; Lustbader & Hamel, 1982). Under some conditions, in fact, we observed a seemingly paradoxical stimulation of polymerization by ddGDP. On closer examination we found that ddGDP itself was able to support microtubule formation in the complete absence of GTP other than the nonexchangeable nucleotide.

Materials and Methods

Materials. Purified calf brain tubulin, heat-treated MAPs, and *Escherichia coli* ribosomes and elongation factor G were prepared as described previously (Hamel, 1975; Hamel & Lin, 1981a; Hamel et al., 1981, 1983). The tubulin was electrophoretically homogeneous, free of nucleoside diphosphatekinase and ATPase activities, freed of unbound nucleotide by gel filtration chromatography, and contained about 1 molar equiv each of GTP and GDP (Hamel & Lin, 1981a). The MAPs contained both MAP-2 and τ factor and were essentially free of GTPase, ATPase, and nucleosidediphosphate kinase activities (Hamel et al., 1981). GTP and ddGTP, obtained from Sigma and P-L, were purified by triethylammonium bicarbonate gradient chromatography on DEAE-Sephadex A-25. Mes (adjusted to pH 6.4 with NaOH), phosphoenolpyruvate, ATP (which was repurified), rabbit muscle pyruvate kinase, yeast inorganic pyrophosphatase, and colchicine were from Sigma; podophyllotoxin was from Aldrich; hog brain GMP kinase was from Boehringer; poly(ethylenimine)–cellulose thin-layer sheets were from Brinkmann; [α-^{32}P]GTP and $^{32}P_i$ were from Amersham. Monosodium glutamate with relatively low Mg^{2+} contamination was prepared from glutamic acid obtained from Schwarz/Mann by addition of NaOH to pH 6.6.

Preparation of ddGDP. A 6-mL reaction mixture containing 44 μmol of ddGTP (obtained from P-L, used without repurification since the major contaminant was ddGDP), 30 mM $MgCl_2$, 80 mM NH_4Cl, 50 mM imidazole hydrochloride (pH 7.4), 10 mM 2-mercaptoethanol, 0.5 mg of elongation factor G, and 11.2 mg of ribosomes was incubated for 24 h at 37 °C. At this point and after 48 h an additional 0.5 mg of elongation factor G and 11.2 mg of ribosomes were added. At 72 h 3 mL of 50% acetic acid was added, and the precipitated ribosomes were removed by centrifugation for 5 min at 20000*g*. The supernatant was lyophilized, and the nucleotide was purified on DEAE-Sephadex A-25 by triethylammonium bicarbonate gradient elution (Figure 1A). The fractions containing ddGDP were pooled, diluted 5-fold, and applied to a second DEAE-Sephadex A-25 column which was developed in the same way (Figure 1B). The fractions on the left and right sides of the peak were pooled separately and lyophilized, and the nucleotide was dissolved in water.

Turbidimetry and Electron Microscopy. Tubulin polymerization was followed turbidimetrically (Gaskin et al., 1974) in a Gilford Model 250 recording spectrophotometer equipped with a Lauda thermostatically controlled water bath. After base lines were established with the water bath at 0 °C, the thermostat was set at 37 °C. The point at which the water bath reached 37 °C was defined as zero time. No turbidity change was observed before this point in the experiments described here. Further details of the temperature equilibration have been presented elsewhere (Hamel et al., 1983).

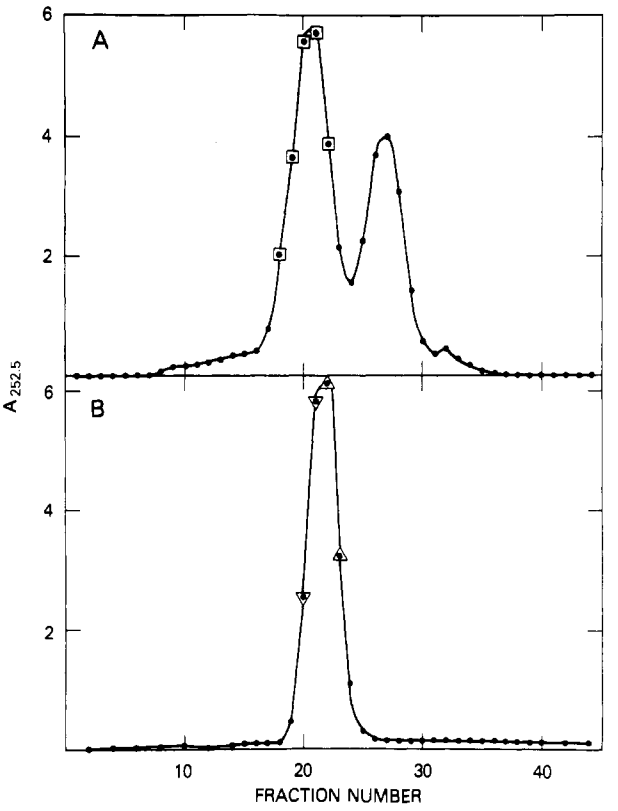

FIGURE 1: Purification of ddGDP. (A) Initial separation of ddGDP and ddGTP by ion-exchange chromatography. After partial degradation of ddGTP as described in the text, the nucleotide mixture was applied to a 1.5 × 10 cm DEAE-Sephadex A-25 column (HCO_3^- form). The column was developed with a 560-mL gradient of triethylammonium bicarbonate from 0.05 to 0.9 M, and fractions of about 12–13 mL were collected. After the $A_{252.5}$ of the fractions was determined, fractions 18–23 were pooled (indicated by boxes) and diluted with 500 mL of water. (B) Repurification of ddGDP. The pooled, diluted fractions from the previously described column were applied to a second column of DEAE-Sephadex A-25 and repurified identically. The fractions from the left-hand (indicated by the inverted triangles) and the right-hand (indicated by the upright triangles) sides of the peak were pooled separately as described in the text.

Electron microscopy was performed as described previously (Hamel et al., 1981, 1982).

Preparation of [β-^{32}P]ddGDP, [β-^{32}P]ddGTP, and [α-^{32}P]GDP. Yeast inorganic pyrophosphatase was used to prepare ddGMP from ddGDP by the method of Schlesinger & Coon (1960). A 5-mL reaction mixture contained 1 mM ddGDP, 2 mM $ZnCl_2$, 0.1 M succinate (pH 5.9), and 500 units of enzyme. It was incubated for 6 h at 37 °C and then boiled for 5 min. The formation of ddGMP was quantitative, and it was purified by triethylammonium bicarbonate gradient chromatography on DEAE-Sephadex A-25.

GMP kinase and pyruvate kinase were used to prepare [β-^{32}P]ddGTP from [γ-^{32}P]ATP [prepared by the method of Glynn & Chappell (1964)], phosphoenolpyruvate, and ddGMP. Reaction conditions were as described previously (Hamel, 1975) for the preparation of [β-^{32}P]dGTP, except that the reaction with hexokinase was eliminated. Instead, the reaction mixture was diluted with 20 mL of 0.1 M $NaIO_4$. After 1.5 h in the dark at room temperature, 2 mL of 20% glucose was added. After another 10 min, 10 mL of 0.4 M $NaBH_4$ was added. The room temperature incubation continued another hour, and the nucleotide was purified by triethylammonium bicarbonate gradient chromatography on DEAE-Sephadex A-25. About 15% of the ^{32}P was in the γ position, so the entire preparation was degraded to [β-^{32}P]-ddGDP with elongation factor G and ribosomes and repurified

as described above but on a smaller scale. A portion of the purified [β-^{32}P]ddGDP was rephosphorylated with phosphoenolpyruvate and pyruvate kinase and repurified. Over 95% of the ^{32}P was now in the β position.

Elongation factor G and ribosomes were also used to prepare [α-^{32}P]GDP from [α-^{32}P]GTP, with purification of the product by triethylammonium bicarbonate gradient chromatography on DEAE-Sephadex A-25.

Nucleotide Hydrolysis. Thin-layer chromatography on poly(ethylenimine)–cellulose in 1.0 M KH_2PO_4 and autoradiography were used to measure nucleotide hydrolysis by following the formation of $^{32}P_i$ or ^{32}P-labeled diphosphates, as appropriate (Hamel & Lin, 1981a). Data are expressed as nanomoles of hydrolytic product formed per milliliter of reaction. Reactions were initiated by transferring the reaction mixtures from an ice bath to a water bath at 37 °C. After the desired incubation, 10 μL of each reaction mixture was added to 20 μL of 25% acetic acid, and 10 μL of the resulting mixture was spotted on the thin-layer sheet. Chromatography was begun while the spots were still wet.

Incorporation of Radioactive Nucleotides into Polymer. Each 0.25-mL reaction mixture contained the indicated components and was incubated for 60 min at 37 °C. They were then centrifuged at 25–30 °C in a Beckman Ti 50 rotor at 40000 rpm for 30 min. The supernatants were discarded, and the pellets were washed twice with 1.0 mL of 0.1 M Mes–0.5 mM $MgCl_2$. The pellets were dissolved in 0.25 mL of 8 M urea, and the protein concentrations and radioactivity of the resulting solutions were determined. The data are expressed as picomoles of nucleotide per microgram of protein in the pellet. To estimate nonspecific entrapment of radioactivity, [γ-^{32}P]ATP was added to duplicate reaction mixtures with nonradiolabeled guanine nucleotides. The amount of radioactivity found in the pellets corresponded to 0.5–1.0 pmol of nucleotide/μg of protein. This may overestimate nonspecific entrapment, however, since evidence has recently been presented that ATP specifically binds to tubulin (Zabrecky & Cole, 1982). Comparable results were obtained with centrifugation of reaction mixtures through a sucrose cushion, except that the protein pellets were smaller.

Analysis of Total Nucleotide Bound to Tubulin after Polymerization with ddGDP. Reaction mixtures (10 mL) were prepared containing 60 μM GTP or ddGDP, 1.0 M glutamate, and 30 mg of tubulin. They were incubated for 90 min at 37 °C and then centrifuged for 30 min in a Beckman Ti 50 rotor at 45000 rpm at room temperature. The pellets were homogenized in 0.4 mL of cold 1.0 M glutamate. After 30 min on ice the suspensions were centrifuged as before, but at 2 °C. The clarified tubulin solutions were separately placed on Sephadex G-50 superfine columns (1.5 × 10 cm) which were equilibrated and developed with 1.0 M glutamate. The protein peaks were pooled separately, and solid urea was added to a final concentration of 8 M. At this point 13.5 mg of tubulin was recovered when GTP was used to support polymerization and 12.6 mg when ddGDP was used. A total of 12.2 mg of protein of each preparation was applied to Sephadex G-50 superfine columns (2.5 × 15 cm) which were equilibrated and developed with 8 M urea. The entire nucleotide peak eluting from each column was pooled, and the $A_{252.5}$ indicated 134 nmol of guanine nucleotide was recovered when GTP was used to support polymerization and 135 nmol when ddGDP was used (8.9 and 9.6 mg of protein were recovered in the protein peaks, respectively, for nucleotide:protein molar ratios of 1.65 and 1.55). A trace amount (about 4 pmol) of [α-^{32}P]GDP was added to each nucleotide pool, as a marker, and they were applied separately to DEAE-Sephadex A-25 columns (1.5 × 10 cm) which were developed with 100 mL of triethylammonium bicarbonate gradients (0.05–1.2 M). Two-milliliter fractions were collected, and their $A_{252.5}$ and radioactivity were determined.

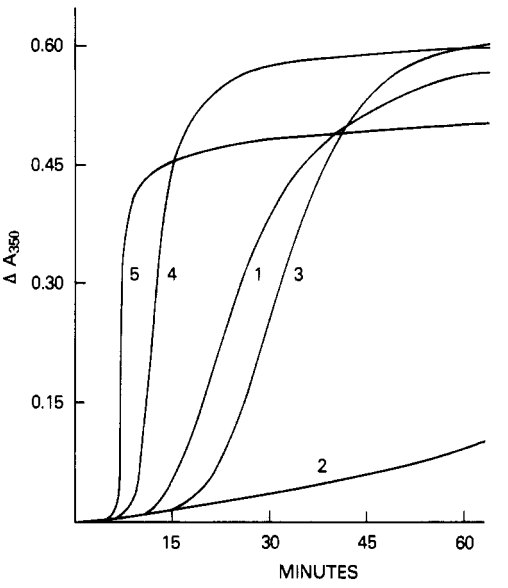

FIGURE 2: Glutamate-induced polymerization of purified tubulin supported by ddGDP. Each 0.2-mL reaction mixture contained 1.0 mg/mL purified tubulin, 1.0 M glutamate, and the indicated nucleotide: curve 1, 10 μM GTP; curve 2, 10 μM ddGDP; curve 3, 30 μM ddGDP; curve 4, 0.1 mM ddGDP; curve 5, 1 mM ddGDP.

Results

Tubulin Polymerization with ddGDP. When preliminary experiments demonstrated that ddGDP was able to induce tubulin polymerization, we were particularly concerned that the activity was due to residual ddGTP: our preparative method is an enzymatic degradation of ddGTP, which is quite active in tubulin polymerization, while ddGDP has minimal inhibitory effects. Repurification, however, did not reduce the activity of any preparation, and no evidence of ddGTP has been found by thin-layer chromatography. Even when fractions of the left and right sides of the ddGDP peak were pooled separately, the two portions had equal activity.

Maximum sensitivity for ddGDP was observed with glutamate-induced polymerization (Figure 2). The minimum effective concentration was 30 μM, with progressively earlier and brisker, but not more extensive, reactions occurring at higher nucleotide concentrations. The reaction observed with 30 μM ddGDP differed little from that with 10 μM GTP, the minimal effective concentration of GTP (Hamel & Lin, 1981b; Lustbader & Hamel, 1982). In glutamate-induced polymerization the threshold concentration of ddGTP is also about 10 μM (Lustbader & Hamel, 1982). If ddGTP were responsible for the activity observed with ddGDP, the level of contamination would be about 30% and should be obvious both on thin-layer chromatography and in the repurification of ddGDP (Figure 1B).

When the activity of ddGDP was examined in MAP-dependent polymerization, a substantially higher nucleotide concentration was required (Figure 3). In 0.1 M Mes–0.5 mM $MgCl_2$ a very sluggish reaction occurred at 0.2 mM ddGDP, with progressively earlier and brisker reactions occurring at higher nucleotide concentrations. As with ddGTP-supported polymerization (Hamel et al., 1983), the turbidity plateau with ddGDP substantially exceeded that with

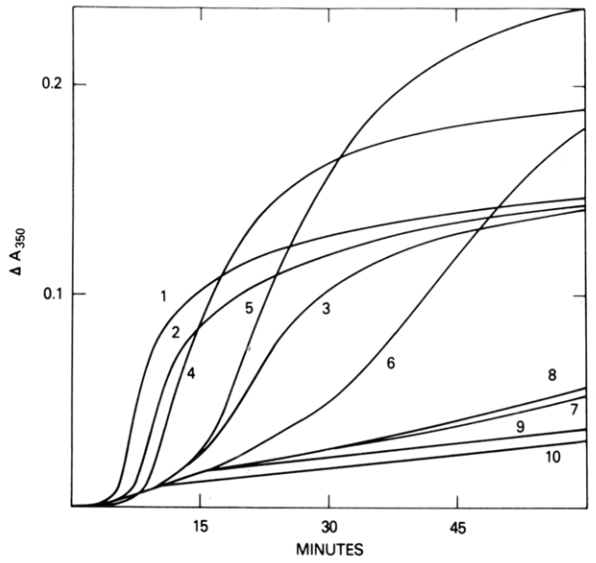

FIGURE 3: MAP-dependent polymerization of tubulin supported by ddGDP. Each 0.2-mL reaction mixture contained 1.0 mg/mL purified tubulin, 0.1 M Mes, 0.5 mM $MgCl_2$, the indicated nucleotide, and, if indicated, 0.2 mg/mL heat-treated MAPs: curve 1, MAPs plus 1 mM GTP; curve 2, MAPs plus 0.1 mM GTP; curve 3, MAPs plus 10 μM GTP; curve 4, MAPs plus 1 mM ddGDP; curve 5, MAPs plus 0.4 mM ddGDP; curve 6, MAPs plus 0.2 mM ddGDP; curve 7, MAPs plus 0.1 mM ddGDP; curve 8, MAPs only (no nucleotide); curve 9, 1 mM GTP only (no MAPs); curve 10, 1 mM ddGDP only (no MAPs).

GTP in the MAP-dependent reaction. Centrifugation studies indicate that this does represent more extensive polymerization than with GTP. With 0.5 mM ddGDP 69% (range 65–75%) of the protein in the reaction mixture was recovered in the pellet, as compared to 49% (range 46–52%) with 0.5 mM GTP. Furthermore, we have determined critical concentrations for tubulin by the dilutional method (Weisenberg & Deery, 1976; Karr et al., 1979) with ddGDP, as well as with ddGTP and GTP (reaction mixtures contained MAPs, 0.1 M Mes, 0.5 mM $MgCl_2$, and 0.4 mM nucleotide). The values obtained were about 0.05 mg/mL with both ddGDP and ddGTP and about 0.1 mg/mL with GTP. With all three nucleotides the amount of unpolymerized protein in the centrifugal assays was significantly higher than the apparent critical concentrations in the dilutional studies. The reason for this is unknown but may be a consequence of our using subsaturating amounts of MAPs in our assays [see Hamel et al. (1983) for a further discussion of this point].

A major difference between polymerization with ddGDP and ddGTP is the requirement for MAPs. With ddGTP, MAP-independent polymerization occurs in 0.1 M Mes–0.5 mM $MgCl_2$ (Hamel et al., 1983). With ddGDP under this reaction condition, there was no significant turbidity development without MAPs (Figure 3). Thus far, at low ionic strengths, we have observed no MAP-independent polymerization with ddGDP.

The morphology of the polymer formed wth ddGDP and both MAPs and glutamate was examined in the electron microscope (Figure 4). With MAPs typical microtubules were the predominant product (Figure 4A). With glutamate open sheets of parallel protofilaments, analogous to those formed with GTP (Hamel et al., 1982), were observed (Figure 4B).

The ddGDP-supported, MAP-dependent reaction displayed properties typical of most tubulin polymerization reactions. Figure 5A demonstrates that microtubule formation was inhibited by the antimitotic drugs podophyllotoxin and colchicine, as well as by Ca^{2+}.[2] The ddGDP-supported reaction

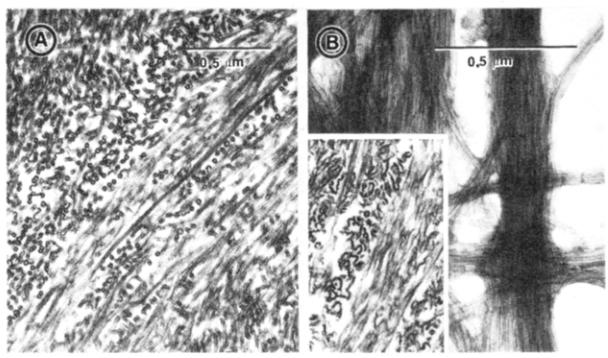

FIGURE 4: Morphology of polymer formed with ddGDP. (A) MAP-dependent reaction. The 0.3-mL reaction mixture contained 1.0 mg/mL purified tubulin, 0.2 mg/mL heat-treated MAPs, 0.1 M Mes, 0.5 mM $MgCl_2$, and 1 mM ddGDP. After 1 h at 37 °C the polymer was harvested by centrifugation, fixed with glutaraldehyde, and stained; a thin section was prepared and examined in the electron microscope (Hamel et al., 1982) (22680×). (B) Glutamate-induced reaction. The 0.1-mL reaction mixture contained 1.0 mg/mL purified tubulin, 1.0 M glutamate, and 1.0 mM ddGDP. After 1 h at 37 °C, 2 μL of the reaction mixture was placed on a 400-mesh carbon-stabilized parlodian-coated grid, negatively stained, and examined in the electron microscope (37800×). (Inset) Polymer was harvested by centrifugation from a similarly prepared 0.3-mL reaction mixture, fixed with formaldehyde (Hamel et al., 1982), and stained; a thin section was prepared and examined in the electron microscope (22680×).

was also exquisitely sensitive to GDP (Figure 5B), significant inhibition occurring with a molar ratio of GDP to ddGDP of 1:100. Comparable inhibition of GTP-supported polymerization in this system requires GDP in 2–3-fold molar excess over GTP (data not presented). Once polymerized, microtubules formed with ddGDP are disrupted by low temperatures and Ca^{2+} (data not presented).

We have found with both glutamate- (Hamel & Lin, 1981b; Lustbader & Hamel, 1982) and MAP-dependent polymerization (data not presented) that GDP displayed maximum inhibitory activity if the Mg^{2+} concentration was minimized, while polymerization was either unaffected or stimulated at higher Mg^{2+} concentrations (Hamel & Lin, 1981a,b; Lustbader & Hamel, 1982; Hamel et al., 1983). MAP-dependent, ddGDP-supported polymerization, in contrast, was found to be reduced at higher Mg^{2+} concentrations. Figure 6 demonstrates that polymerization with 0.2 mM ddGDP was markedly enhanced if Mg^{2+} was omitted from the reaction mixture, while polymerization with 10 μM GTP (the minimum effective concentration) was unaffected.[3] The threshold concentration

[2] We have routinely observed a nucleotide-independent increase in turbidity in our reaction mixtures (Hamel et al., 1981, 1983). This reaction is more rapid at higher temperatures, and once initiated, it is linear for long periods of time. The nucleotide-independent reaction is not cold reversible, and it is more rapid with MAPs than without MAPs (see Figure 3). It is not a true base-line reaction: if GTP-dependent and independent turbidity development are followed simultaneously and the reaction temperature is then reduced to 0 °C, turbidity in the reaction mixture with GTP invariably drops below that in the reaction mixture without GTP [Figure 4 (Hamel et al., 1981)]. No discrete structures have been observed when incubated reaction mixtures without nucleotide are centrifuged and the pellets examined in the electron microscope [Figure 6F (Hamel et al., 1983)]. The reaction thus appears to primarily represent aggregation, although we cannot at this point exclude some ring formation. We believe that turbidity development in the inhibited reactions of Figure 5A, particularly those represented by curves 3 (podophyllotoxin) and 4 (calcium), is caused largely or entirely by the nucleotide-independent aggregation reaction. Calcium will cause complete depolymerization of microtubules formed with ddGDP, and electron microscopic examination of a reaction mixture containing podophyllotoxin failed to demonstrate discrete structures (data not presented).

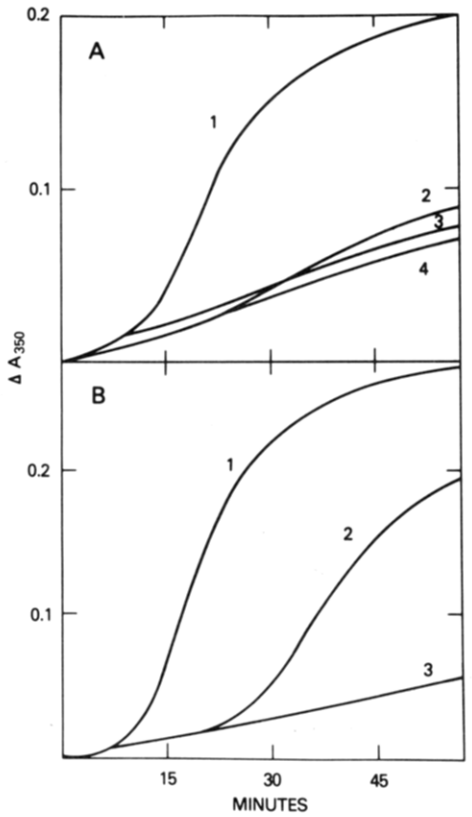

FIGURE 5: Inhibition of ddGDP-supported, MAP-dependent polymerization. Each 0.2-mL reaction mixture contained 1.0 mg/mL purified tubulin, 0.2 mg/mL heat-treated MAPs, 0.1 M Mes, 0.5 mM $MgCl_2$, 0.5 mM ddGDP, and other components as indicated. (A) Inhibition by antimitotic drugs and Ca^{2+}. Curve 1, no further addition; curve 2, 20 μM colchicine; curve 3, 20 μM podophyllotoxin; curve 4, 2 mM $CaCl_2$. (B) Inhibition by GDP. Curve 1, no further addition; curve 2, 5, μM GDP; curve 3, 10 μM GDP.

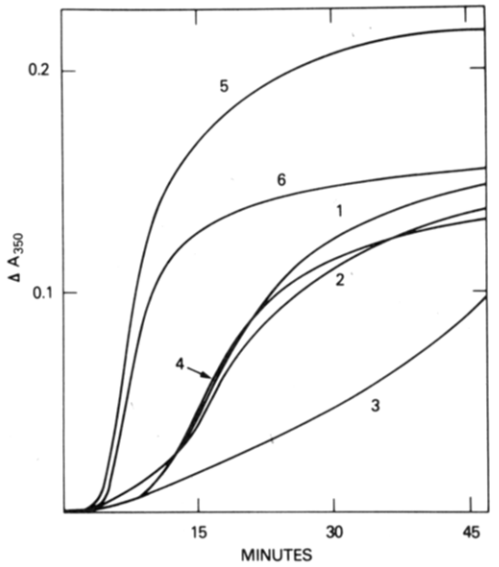

FIGURE 6: Enhancement of ddGDP-supported, MAP-dependent polymerization at low Mg^{2+} concentrations. Each 0.2-mL reaction mixture contained 1.0 mg/mL purified tubulin, 0.2 mg/mL heat-treated MAPs, 0.1 M Mes, and nucleotides and $MgCl_2$ as indicated: curve 1, 10 μM GTP plus 0.5 mM $MgCl_2$; curve 2, 10 μM GTP only; curve 3, 0.2 mM ddGDP plus 0.5 mM $MgCl_2$; curve 4, 0.2 mM ddGDP only; curve 5, 1 mM ddGDP only; curve 6, 1 mM GTP only.

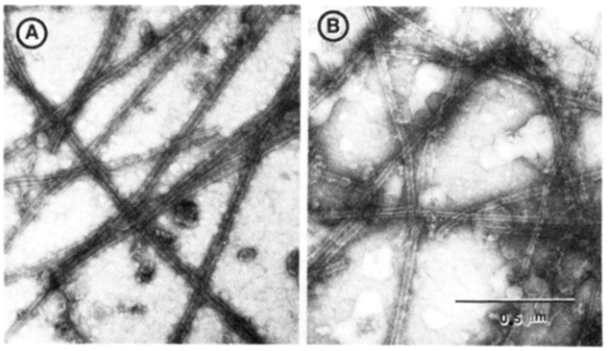

FIGURE 7: Microtubules formed with ddGDP in the presence (A) or absence (B) of Mg^{2+}. Reaction mixtures containing 1 mg/mL tubulin, 0.2 mg/mL heat-treated MAPs, 1 mM ddGDP, 0.1 M Mes, and, if indicated, 0.5 mM $MgCl_2$ were incubated for 45 min at 37 °C. The reaction mixtures were diluted with four parts of isothermic 50% sucrose solutions (Terry & Purich, 1980) containing 0.1 M Mes and, if appropriate, 0.5 mM $MgCl_2$. Negatively stained specimens were prepared and examined in the electron microscope. Magnification 32130×.

of ddGDP was reduced to about 0.1 mM without Mg^{2+}, but MAPs were still required. Moreover, if Mg^{2+} was omitted at high nucleotide concentrations, all parameters of the ddGDP-supported reaction exceeded those of the GTP-supported reaction: with ddGDP, polymerization had an earlier onset, had a faster rate, and was more extensive (Figure 6, curves 5 and 6). The omission of Mg^{2+} did not affect polymer morphology, however. Reaction mixtures with 1 mM ddGDP with and without added Mg^{2+} were examined in the electron microscope, and the polymer in both was found to consist of microtubules (Figure 7).

No Nucleotide Hydrolysis Occurs. The finding that ddGDP supports tubulin polymerization raises several questions: (1) Is polymerization indirectly supported via an unknown contaminating enzyme that can generate GTP from exchangeably bound GDP and ddGDP? (2) Is ddGDP hydrolyzed? (3) Is ddGDP incorporated into the polymer? (4) Does ddGDP trigger hydrolysis of the molar equivalent of GTP bound (presumably nonexchangeably) in our tubulin preparation and thereby induce polymerization?

To answer these questions [β-^{32}P]ddGTP and [β-^{32}P]ddGDP were synthesized and their interactions with tubulin compared to those of [α-^{32}P]GTP. We found no hydrolysis of ddGDP, nor any evidence that it was altered, in incubations as long as 2 h at 37 °C. GTP hydrolysis required both tubulin and

Table I: Stability of [β-^{32}P]ddGDP during Polymerization[a]

additions	nmol of nucleotide hydrolyzed
[β-^{32}P]ddGDP + tubulin + MAPs	0[b]
[α-^{32}P]GTP + tubulin	1.6[c]
[α-^{32}P]GTP + tubulin + MAPs	60.9[c]
[β-^{32}P]ddGTP + tubulin	180.5[d]
[β-^{32}P]ddGTP + tubulin + MAPs	113.0[d]

[a] Each 50-μL reaction mixture was incubated for 2 h at 37 °C and contained 0.1 M Mes, 0.5 mM $MgCl_2$, 1 mg/mL tubulin, and, if indicated, 0.2 mg/mL heat-treated MAPs and the indicated nucleotide at 0.5 mM. [b] The amount of P_i formed was determined. [c] The amount of [α-^{32}P]GDP formed was determined. [d] The amount of [β-^{32}P]ddGDP formed was determined.

[3] The solutions of Mes and glutamate [in which the tubulin and MAPs were stored (Hamel & Lin, 1981a; Hamel et al., 1981)] used here have been analyzed by atomic absorption spectroscopy and would contribute residual Mg^{2+} to the reaction mixtures of about 30 μM. In addition, heat-treated MAPs and tubulin were subjected to gel filtration chromatography in Mg^{2+}-depleted 0.1 M Mes and similarly analyzed. No significant Mg^{2+} was found in the MAPs, but up to 2 molar equiv of the cation remained associated with the tubulin.

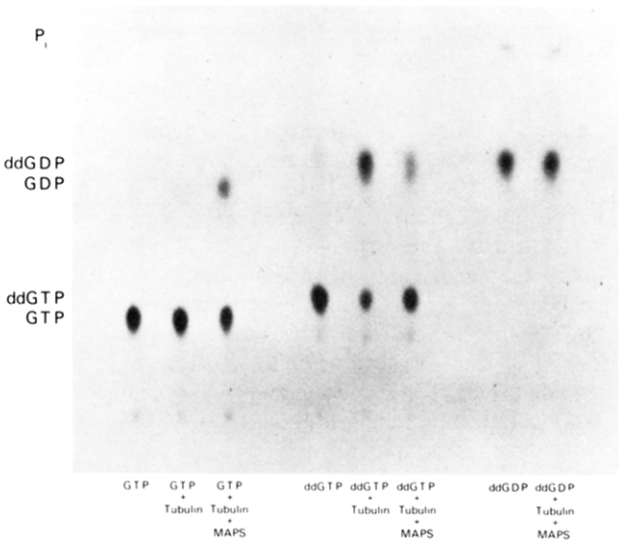

FIGURE 8: Stability of ddGDP with tubulin and MAPs. Each 50-μL reaction mixture contained 0.1 M Mes, 0.5 mM $MgCl_2$, and the following, as indicated in the figure: 1.0 mg/mL purified tubulin, 0.2 mg/mL heat-treated MAPs, 0.5 mM [α-^{32}P]GTP, 0.5 mM [β-^{32}P]ddGTP, and 0.5 mM [β-^{32}P]ddGDP. After 2 h at 37 °C 10 μL of each reaction mixture was added to 20 μL of 25% acetic acid, and 10 μL of each mixture was spotted on a 10 × 10 cm poly(ethylenimine)–cellulose thin-layer sheet. While the spots were still wet, chromatography was begun in 1.0 M KH_2PO_4. After chromatography was complete, the thin-layer sheet was dried and an autoradiogram prepared with Kodak XRP-1 X-ray film.

Table II: Incorporation of ddGDP into Microtubules[a]

nucleotide added	pmol of nucleotide/ μg of protein in pellet
0.1 mM [α-^{32}P]GTP	8.3
0.2 mM [β-^{32}P]ddGDP	8.4
0.2 mM [β-^{32}P]ddGDP – $MgCl_2$	9.4

[a] Reaction mixtures contained 0.1 M Mes, 0.5 mM $MgCl_2$ except where indicated, 1.0 mg/mL tubulin, 0.2 mg/mL heat-treated MAPs, and the indicated nucleotide. Other experimental details are given in the text.

MAPs, while ddGTP was hydrolyzed both with tubulin alone or with tubulin plus MAPs [these reactions will be described in greater detail elsewhere (unpublished results)]. The autoradiogram presented in Figure 8, together with the data in Table I derived from the corresponding thin-layer plate, demonstrates these findings. In particular the stability of [β-^{32}P]ddGDP, whether formed from [β-^{32}P]ddGTP or present initially, should be emphasized, for it excludes both hydrolysis of the nucleotide and its serving as a phosphate donor for the generation of GTP.

MAP-dependent microtubules formed in the presence of [α-^{32}P]GTP and [β-^{32}P]ddGDP (both with and without added Mg^{2+}) were harvested by centrifugation. Near stoichiometric, and similar, amounts of nucleotide were found in the pellets (Table II). The bound radioactivity was examined by thin-layer chromatography and autoradiography and was 92% GDP after polymerization with GTP and 88% ddGDP after polymerization with ddGDP.

Glutamate-induced polymerization was used to approach the question of the fate of the nonexchangeable GTP, since low concentrations of ddGDP support the reaction. We have demonstrated elsewhere that GTP-supported, glutamate-induced polymerization is associated with GTP hydrolysis (Hamel & Lin, 1981a,b; Hamel et al., 1982). Figure 9A demonstrates the nucleotide content of tubulin after GTP-

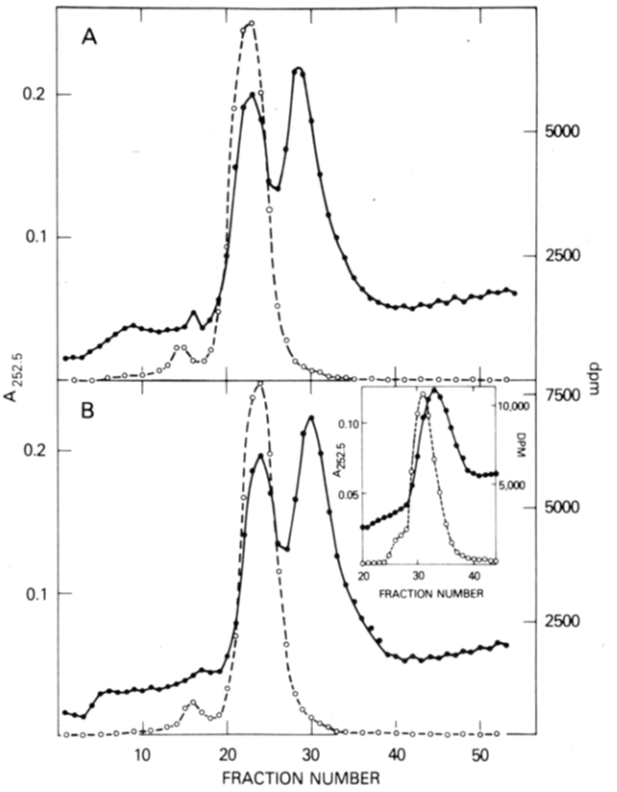

FIGURE 9: Nonexchangeable GTP is unaltered in glutamate-induced, ddGDP-supported tubulin polymerization. Reaction conditions and methodology are described in detail in the text. $A_{252.5}$ (●); radioactivity (dpm) (○). (A) Tubulin-bound nucleotide after GTP-supported polymerization. (B) Tubulin-bound nucleotide after ddGDP-supported polymerization. (Inset) Resistance of the diphosphate peak, after ddGDP-supported polymerization, to periodate and borohydride as compared to exogenously added [α-^{32}P]GDP. Nucleotide bound to tubulin after polymerization with ddGDP was obtained as described in the text, except that the initial polymerization reaction contained twice as much tubulin and ddGDP. The diphosphate peak was purified to homogeneity by two additional passages on DEAE-Sephadex A-25 and lyophilized to dryness. The residue was dissolved in 0.25 mL of 0.25 M $NaIO_4$ and left 24 h at room temperature in the dark. Fifty microliters of 6 M LiCl and 1.8 mL of 1 M LiCl in ethanol at −20 °C were added sequentially to the reaction mixture. A small precipitate was collected 8 h later, washed twice with ethanol, dried in vacuo, and dissolved in 1 mL of 0.5 M $NaBH_4$. After 24 h at room temperature in the dark, the reaction mixture was diluted 8-fold and applied to a 1.5 × 10 cm DEAE-Sephadex A-25 column which was developed with a 100-mL triethylammonium bicarbonate gradient (0.05–0.9 M). Two-milliliter fractions were collected, and their $A_{252.5}$ and radioactivity were determined.

supported polymerization and depolymerization with removal of unbound nucleotide. We found 1.65 mol of nucleotide bound per mol of tubulin, 59% GTP and 41% GDP. When ddGDP was used to support polymerization (Figure 9B), 1.55 mol of nucleotide per mol of tubulin was obtained, 62% GTP and 38% ddGDP. The near equivalence of the GTP recovered demonstrates that hydrolysis of the nonexchangeable GTP does not occur in glutamate-induced, ddGDP-supported polymerization.

The inset of Figure 9B demonstrates that the diphosphate obtained from tubulin polymerized with ddGDP was in fact ddGDP. The diphosphate peak was repurified and treated sequentially with periodate and borohydride, a procedure which converts GDP quantitatively to a product which elutes at a lower salt concentration than GDP from DEAE-Sephadex A-25 (Hamel, 1975). The exogenously added marker [α-^{32}P]GDP was now distinct from the diphosphate peak isolated from the polymerized tubulin, eluting from the DEAE-Sephadex A-25 column earlier than the nonradioactive peak.

Discussion

The initial evidence that GTP hydrolysis was not essential for microtubule assembly was the finding that the nonhydrolyzable GTP analogues guanosine 5′-(β,γ-methylenetriphosphate) and guanosine 5′-(β,γ-imidotriphosphate) supported the reaction (Weisenberg et al., 1976; Arai & Kaziro, 1976; Sutherland, 1976; Penningroth & Kirschner, 1977). It was subsequently reported that GDP would permit the elongation of microtubule seeds, but not de novo polymerization (Carlier & Pantaloni, 1978; Karr et al., 1979). It was therefore suggested that nucleation required a triphosphate in the exchangeable site. Almost simultaneously, however, the nonhydrolyzable GDP analogue p(CH_2)pG was reported to support a weak polymerization reaction (Sandoval et al., 1978). No turbidity development occurred with p(CH_2)pG, but the nucleotide caused a small portion of both tubulin and microtubule protein to sediment when centrifuged at 48000g. The pellet consisted in part of short microtubules. It was next found that the drug taxol promoted efficient microtubule formation in the absence of GTP: the drug induced both nucleation and elongation with GDP in the exchangeable site (Schiff & Horwitz, 1981; Gaskin, 1981; Hamel et al., 1981).

We have now found that ddGDP, a ribose-modified hydrolyzable analogue of GDP, is able to promote tubulin polymerization with either MAPs or high concentrations of glutamate. Although the minimum nucleotide concentration required to obtain a reaction was significantly higher with ddGDP than with either GTP or ddGTP, efficient polymerization occurred at high ddGDP concentrations. With MAPs, at these higher ddGDP levels, polymerization was significantly more extensive with the analogue than with GTP, and under optimum conditions for the ddGDP-supported reaction (1 mM nucleotide, no Mg^{2+}), all its parameters exceeded those of the GTP-supported reaction—the lag period was shorter, and the reaction rate and extent were greater. In short, ddGDP can support effective nucleation and elongation and, if bound in the exchangeable site, appears to lower the critical concentration of tubulin required for polymerization.

Thus far these properties are unique to ddGDP. We have seen no significant turbidity development, with or without Mg^{2+}, with any other nucleoside diphosphate or GDP analogue we have examined, including ADP, 2′,3′-dideoxyadenosine 5′-diphosphate, and p(CH_2)pG.[4]

With MAPs typical microtubules were formed, and ddGDP was completely stable in the reaction mixture. Moreover, at least in 1.0 M glutamate, the nonexchangeable GTP is not altered in the polymerization process.

The most reasonable interpretation of these findings is that, in binding to the exchangeable site, some subtle feature of the conformation of ddGDP or of the nucleotide–protein interaction converts tubulin from a nonpolymerizable to a polymerizable form. Most intriguing would be the possibility that single tubulin molecules might act as nucleation centers. Whatever the precise molecular mechanism, however, the efficient polymerization we have observed with ddGDP indicates that the γ-phosphate group, as well as its hydrolysis, is not required in either nucleation or elongation under commonly used reaction conditions (0.1 M Mes–0.5 mM $MgCl_2$) and at low tubulin concentrations (1 mg/mL).

The marked enhancement of MAP-dependent ddGDP-supported polymerization by omission of Mg^{2+} is also unique in our experience. With GTP and GTP analogues we have observed either no effect or stimulation of polymerization by Mg^{2+} (Hamel & Lin, 1981a,b; Lustbader & Hamel, 1982; Hamel et al., 1983). The apparent enhancement of the ddGDP–tubulin interaction at low Mg^{2+} concentrations resembles the significant increase in the inhibitory effect of GDP and GDP analogues on polymerization at similar minimal Mg^{2+} concentrations which we have previously reported (Hamel & Lin, 1981b; Lustbader & Hamel, 1982). This common effect of Mg^{2+} in apparently reducing the affinity of the nucleotide for tubulin suggests that ddGDP is interacting with the protein primarily as a GDP analogue rather than as a GTP analogue, even though it stimulates rather than inhibits polymerization.

The ddGDP-supported polymerization reaction is potently inhibited by GDP, a molar ratio of GDP to ddGDP of 1:50 completely suppressing the reaction. This, together with the relatively high concentrations of ddGDP required for polymerization, indicates that the analogue actually has a low affinity for tubulin compared to GDP. This has been confirmed with [β-^{32}P]ddGDP (unpublished results).

Finally, a number of investigators have suggested that GTP hydrolysis may affect the stability of microtubules (Weisenberg et al., 1976; Arai & Kaziro, 1976) and the ability of tubulin dimers to participate in the treadmill reaction (Terry & Purich, 1980; Margolis, 1981; Deery & Weisenberg, 1981; Cote & Borisy, 1981). Since microtubules bearing ddGDP can be formed with tubulin and MAPs by using either ddGTP (with hydrolysis) or ddGDP (without hydrolysis), a direct examination of the effects of nucleotide hydrolysis on the properties of microtubules should be possible.

Registry No. ddGDP, 84328-12-1; 2-(*N*-morpholino)ethanesulfonate, 4432-31-9; glutamic acid, 56-86-0; GTP, 86-01-1; Ca, 7440-70-2; Mg, 7439-95-4; GDP, 146-91-8; ddGTP, 68726-28-3; inorganic pyrophosphatase, 9024-82-2; ddGMP, 85956-71-4; GMP kinase, 9026-59-9; pyruvate kinase, 9001-59-6; [β-^{32}P]ddGTP, 85976-62-1; [γ-^{32}P]ATP, 2964-07-0; phosphoenolpyruvate, 138-08-9; [β-^{32}P]ddGDP, 85976-63-2; [α-^{32}P]GDP, 85956-72-5; [α-^{32}P]GTP, 5087-49-0.

[4] Other GDP analogues without activity are dGDP, 3′-deoxyguanosine 5′-diphosphate, 9-β-D-arabinofuranosylguanine 5′-diphosphate, acycloguanosine diphosphate, the dialcohol derivative of GDP obtained by periodate oxidation and borohydride reduction, 2′-*O*-methylguanosine 5′-diphosphate, 3′-*O*-methylguanosine 5′-diphosphate, guanosine 2′-monophosphate 5′-diphosphate, and guanosine 3′-monophosphate 5′-diphosphate. The highest nucleotide concentration used in all cases was 1 mM.

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Solution Conformation of Asparagine-Linked Oligosaccharides: $\alpha(1-2)$-, $\alpha(1-3)$-, $\beta(1-2)$-, and $\beta(1-4)$-Linked Units†

Jean-Robert Brisson and Jeremy P. Carver*

ABSTRACT: The solution conformation is presented for representatives of each of the major classes of asparaginyl oligosaccharides. In this report the conformation of $\alpha(1-3)$-, $\alpha(1-2)$-, $\beta(1-2)$-, and $\beta(1-4)$-linked units is described. The conformational properties of these glycopeptides were determined by high-resolution ^1H nuclear magnetic resonance in conjunction with potential energy calculations. The NMR parameters that were used in this analysis were chemical shifts and nuclear Overhauser enhancements. Potential energy calculations were used to evaluate the preferred conformers available for the different linkages in glycopeptides and to draw conclusions about the behavior in solution of these molecules. It was found that the linkage conformation of the Manα1-3 residues was not affected by substitution either at the 2-position by αMan or βGlcNAc or at the 4-position by βGlcNAc or by the presence of a bisecting GlcNAc on the adjacent βMan residue.

Asparagine-linked oligosaccharides are structurally similar in that most contain the same mannotriose unit; however, they differ in the pattern of substitution of this core and can be classified according to these different patterns (Carver & Grey, 1981). This variability in structure makes them ideally suited for their postulated role as specific recognition signals on the cell surface (Hughes & Sharon, 1978). Although the behavior of these glycopeptides toward lectins (Goldstein & Hayes, 1978), glycosidases, and transferases has been well documented in recent years (Schachter & Roseman, 1980), most of these interactions have only been characterized in terms of the oligosaccharide primary structure. In order to fully understand the molecular basis of these recognition events, the three-dimensional structure of the carbohydrate chains of glycopeptides must be determined.

The synthesis of Asn-linked oligosaccharides is regulated by a highly specific sequence of events. The type of carbohydrate chain that is eventually synthesized appears to be controlled by a complex interaction between the polypeptide sequence and the relative levels of activity of certain transferases. For example, two well-characterized enzymes are GlcNAc-transferase I and GlcNAc-transferase III (Harpaz & Schachter, 1980; Narasimhan, 1982). The action of the former is an essential requirement for processing of the carbohydrate chain to occur. The action of the GlcNAc-transferase III, on the other hand, is highly inhibitory to the subsequent action of at least four other enzymes in the pathway despite the fact that they act at sites remote from the point of substitution by GlcNAc-transferase III. Thus, it is essential to determine the three-dimensional structure of these substrates

† From the Departments of Medical Genetics and Medical Biophysics, University of Toronto, Toronto, Ontario, Canada M5S 1A8. *Received February 24, 1983.* This research was supported by grants from the Medical Research Council of Canada (MT-3732 and MA-6499) and a studentship (J.-R.B.).

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