Effect of GTP on the dolichol pathway for protein glycosylation in rat liver microsomes

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Incubation of native rat liver microsomes with GTP resulted in enhanced incorporation of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc into lipid acceptors. The stimulation of GlcNAc transfer by GTP was specific for GTP; ATP exerted no effect. The GTP effect was blocked by a non-hydrolysable GTP analogue guanosine 5′-[$\beta\gamma$-imido]triphosphate, indicating that GTP hydrolysis was crucial. Though dolichyl pyrophosphate *NN′*-diacetylchitobiose [Dol-*PP*-(GlcNAc)$_2$] was the main radiolabelled product formed upon incubation of GTP-treated microsomes with UDP-GlcNAc, GTP selectively stimulated UDP-GlcNAc:dolichyl phosphate (Dol-*P*) *N*-acetylglucosaminyl 1-phosphotransferase (*N*-acetylglucosaminyl 1-phosphotransferase). This conclusion was reached on the basis of experiments in which tunicamycin was used to selectively inhibit *N*-acetylglucosaminyl 1-phosphotransferase. The enhanced transformation of Dol-*P* to dolichyl pyrophosphate *N*-acetylglucosamine (Dol-*PP*-GlcNAc) by GTP ultimately led to enhanced protein glycosylation. GTP-induced stimulation of GlcNAc incorporation in lipid and protein by GTP was observed also in microsomes fully permeabilized with *Staph. aureus* α-toxin. These findings refute the previous proposal [Godelaine, Beaufay, Wibo and Ravoet (1983) J. Cell Biol. **97**, 340–350] that increased membrane permeability constitutes the mechanism whereby GTP activates the reactions of the dolichol pathway.

INTRODUCTION

Guanine nucleotides serve a fundamental regulatory function in the control of a wide variety of biochemical processes (Hall, 1990; Bourne et al., 1991; Kaziro et al., 1991). In the ER (endoplasmic reticulum), diverse GTP-dependent functions have been described, including protein transport across the ER membrane (Rapoport, 1992) and intracellular membrane-trafficking processes in the secretory pathway (Balch, 1989; Newman and Ferro-Novick, 1990; Plutner et al., 1991; Rexach and Schekman, 1991; Tan et al., 1992).

It has been reported that in rat liver microsomes, GTP (i) stimulates core glycosylation (Godelaine et al., 1977, 1979a,b, 1983; Godelaine and Beaufay, 1983); (ii) is implicated in regulation of Ca^{2+} transport (Dawson, 1985; Dawson et al., 1986; Henne and Söling, 1986; Nichitta et al., 1987); (iii) changes membrane permeability (Godelaine et al., 1983; Nichitta et al., 1987) and (iv) induces fusion of membranes (Paiement et al., 1980, 1987; Paiement and Bergeron, 1983; Comerford and Dawson, 1988, 1989). These effects are, however, conditioned by the removal of the ribosomes or by the presence of poly(ethylene glycol). Coincidence between these structural and metabolic events has been reported (Dawson et al., 1987; Comerford and Dawson, 1988, 1989; Dawson and Comerford, 1989; Paiement and Bergeron, 1983).

The mechanism by which GTP produces *in vitro* activation remains unknown. It has been suggested that GTP might activate dolichol-mediated glycosylation reactions by changing the microsomal membrane permeability barrier which prevents access of sugar-nucleotides to the lumen of the microsomal vesicles (Godelaine et al., 1983). Likewise, enhanced membrane permeability for Ca^{2+}, which accompanies vesicle fusion, has been incriminated as the mechanism of GTP-dependent Ca^{2+} mobilization from rat liver microsomes (Nichitta et al., 1987).

In an accompanying paper (Bossuyt and Blanckaert, 1993) we present evidence that permeation of sugar-nucleotide into the lumen of the ER is not the rate-limiting factor for reactions involved in the sugar transfer to dolichyl phosphate (Dol-*P*). Hence, the proposal that alteration of membrane permeability constitutes the mechanism of GTP action on core glycosylation requires reappraisal.

The present study was undertaken to characterize the effects of GTP on the *N*-acetylglucosamine (GlcNAc) incorporation in native well-sealed microsomes. This study embodies a description of the kinetics of the GlcNAc incorporation in control and in GTP-pretreated microsomes and addresses the question whether GTP stimulates a well-defined step. The effect of GTP on membrane permeability was assessed, *inter alia*, by investigating whether GTP remains active in microsomes made freely permeable for nucleotide-sugars by pretreatment with the pore-forming *Staph. aureus* α-toxin. Our results refute the idea that GTP enhances *N*-glycosylation by breaching the membrane permeability barrier.

MATERIALS AND METHODS

Chemicals

GTP sodium salt came from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NAD^+ and guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG; 'GMP-PNP') tetralithium salt were obtained from Boehringer Mannheim Biochemicals. Sources of the other chemicals are indicated in the accompanying paper (Bossuyt and Blanckaert, 1993).

Abbreviations used: CHAPSO, 3-[(3-chloramidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate; Dol-*P*, dolichyl phosphate; Dol-*PP*-GlcNAc, dolichyl pyrophosphate *N*-acetylglucosamine; Dol-*PP*-(GlcNAc)$_2$, dolichyl pyrophosphate *NN′*-diacetylchitobiose; *N*-acetylglucosaminyltransferase, UDP-GlcNAc:Dol-*PP*-GlcNAc *N*-acetylglucosaminyltransferase (EC 2.4.1.141); *N*-acetylglucosaminyl 1-phosphotransferase, UDP-GlcNAc:Dol-*P* *N*-acetylglucosaminyl 1-phosphotransferase (EC 2.7.8.15); ER, endoplasmic reticulum; PtdCho, L-α-phosphatidylcholine; p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate ('GMP-PNP') tetralithium salt.

Preparation and treatment of microsomes

Preparation of rat liver microsomes, assessment of their structural integrity and membrane permeabilization by *Staph. aureus* α-toxin were done as outlined in the companion paper (Bossuyt and Blanckaert, 1993).

Dol-*P* glycosyltransferase assay

Incorporation of GlcNAc into Dol-*PP*-GlcNAc, Dol-*PP*-$(GlcNAc)_2$ and protein was measured as described in the accompanying paper (Bossuyt and Blanckaert, 1993). Exogenous Dol-*P* was supplemented via L-α-phosphatidylcholine (PtdCho) liposomes, which were prepared as described (Bossuyt and Blanckaert (1993). Likewise, some experiments were carried out in which the ATP-regenerating system (3 mM ATP, 7 mM phosphocreatine and 0.05 mg/ml creatine kinase) was omitted from the incubation mixture. Under selected circumstances, the reaction medium was supplemented with GTP, p[NH]ppG, GDP-Man, UDP-Glc, α-lactalbumin or tunicamycin.

Incorporation of radiolabel in trichloroacetic acid-precipitable material was measured as described by Godelaine et al. (1977).

Chromatographic analysis of reaction products

The chloroform/methanol extract containing the dolichol derivatives was concentrated in a stream of nitrogen. An aliquot was applied to a silica-gel-coated t.l.c. plate (Merck, Darmstadt, Germany), which was developed in chloroform/methanol/water (60:35:6, by vol). Lanes (each 3 mm wide) were scraped off and analysed for radioactivity. R_F values for Dol-*PP*-GlcNAc and Dol-*PP*-$(GlcNAc)_2$ were in agreement with previously reported values (Elting and Lennarz, 1982).

Other assays and analyses

Radioassay of mannose-6-phosphatase activity and the measurements of total protein concentration were performed as described in the accompanying paper (Bossuyt and Blanckaert, 1993).

Statistical analysis

All results are expressed as means ± S.E.M. for the number of experiments indicated. Statistical analysis was carried out by Student's *t* test.

RESULTS AND DISCUSSION

GTP stimulates the incorporation of GlcNAc into lipid acceptors in microsomes

Table 1 shows activity of GlcNAc incorporation in lipid acceptors when microsomes were incubated under various conditions with UDP-[^{3}H]GlcNAc. Pretreatment of the microsomes with GTP resulted in increased formation of Folch-extractable radiolabel. Addition of ATP-regenerating system alone did not stimulate GlcNAc incorporation into lipid acceptors, indicating a high specificity for GTP. Addition of p[NH]ppG, a non-hydrolysable GTP analogue, to the incubation medium prior to GTP completely blocked the GTP-dependent stimulation of GlcNAc transfer into lipid. This indicates that hydrolysis of the nucleotide triphosphate was pivotal in the stimulation brought about by GTP. GTP-concentration-dependence of GlcNAc incorporation into microsomes is displayed in Figure 1. A near-maximal effect of GTP was reached at 50 μM.

Effect of Dol-*P* concentration on formation of lipid-linked *N*-acetylglucosamine glycosides

Endogenous Dol-*P* in microsomes is far below the saturating concentration for glycosyltransferase activity. The classical approach is to supplement the assay medium with Dol-*P* solubilized in detergent. However, detergent disrupts the microsomal membrane. To preserve integrity of the microsomal vesicles, we selected an alternative approach in which we supplemented the isoprenoid acceptor substrate to the reaction mixture via PtdCho liposomes. This enabled us to optimize enzymic activity while preserving the integrity of the microsomal permeability barrier. Mannose-6-phosphatase latency in the presence of the Dol-*P*-containing liposomes was > 95%, which did not differ from the values found in control microsomes, tested in the absence of liposomes.

The influence of Dol-*P* on GlcNAc incorporation in control and in GTP-pretreated microsomes was investigated. As revealed in Figure 2, Dol-*P* augmented enzymic activity under both

Table 1 Incorporation of GlcNAc into lipid acceptors under various conditions in microsomes

Microsomes were suspended in incubation medium, containing exogenous Dol-*P* (40 μg/ml), but devoid of an ATP-regenerating system. A first preincubation of 10 min was carried out in the absence (conditions A, B, and C) or presence (condition D) of 200 μM p[NH]ppG. Thereafter, either ATP-regenerating system alone (condition B) or ATP-regenerating system with 50 μM GTP (conditions C and D) was added, and incubation at 37 °C was continued for 30 min. Following this total 40 min preincubation period, the transferase assay was started by adding UDP-[^{3}H]GlcNAc. Each value represents the mean ± S.E.M. for four microsomal preparations with duplicate measurements for each. When indicated by an asterisk, the mean is statistically significant different ($P < 0.005$) as compared with the mean obtained under condition A.

Condition	GlcNAc incorporation (pmol/min per mg of protein)
A	33 ± 3
B	33 ± 3
C	87 ± 11*
D	31 ± 3

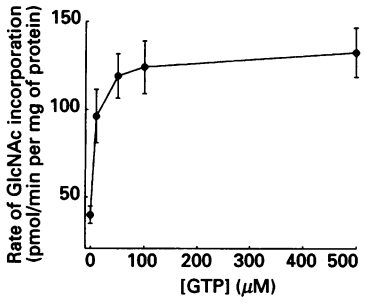

Figure 1 Dependence of the increase in GlcNAc incorporation on concentration of GTP

Microsomes were preincubated for 30 min at 37 °C in incubation mixtures supplemented with various concentrations of GTP, as specified along the abscissa. Reactions were started by addition of UDP-[^{3}H]GlcNAc. GlcNAc incorporation was measured as described in the companion paper (Bossuyt and Blanckaert, 1993). Each point represents the mean ± S.E.M. for four microsomal preparations.

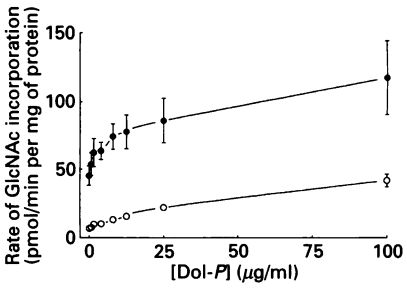

Figure 2 Influence of exogenous Dol-*P* on microsomal GlcNAc incorporation into lipid-linked glycosides

Native microsomes were preincubated for 30 min at 37 °C with various concentrations of exogenous Dol-*P*, supplemented via PtdCho liposomes, in the absence (○) or presence of 50 μM GTP (●). Reactions were started by addition of UDP-[^{3}H]GlcNAc, and initial rates were estimated as described in the companion paper (Bossuyt and Blanckaert, 1993). Each point represents the mean ± S.E.M. for four microsomal preparations.

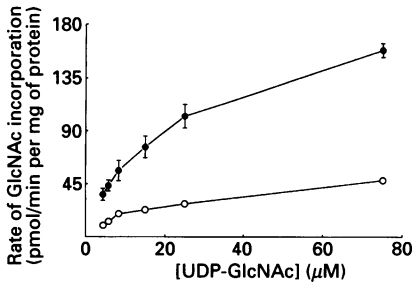

Figure 3 Dependence on the UDP-GlcNAc concentration of the rate of microsomal GlcNAc transfer to Dol-*PP* derivatives for control and GTP-treated microsomes

Initial rate of GlcNAc incorporation into lipid was assessed in native microsomes that had been preincubated in the absence (○) or presence (●) of 50 μM GTP for 30 min at 37 °C in incubation mixtures supplemented with 10 μg of exogenous Dol-*P*/ml. Reactions were started by adding various UDP-[^{3}H]GlcNAc concentrations ranging from 3.9 to 75 μM. Each point represents the mean ± S.E.M. for four microsomal preparations.

conditions, i.e. in the presence as well as in the absence of GTP. Small amounts of exogenous Dol-*P* (< 8 μg/ml) caused a markedly larger increase of GlcNAc incorporation for GTP-treated microsomes as compared with control microsomes without GTP. Consequently the degree of stimulation by GTP was markedly dependent on the concentration of exogenous Dol-*P*, the stimulation being more pronounced in the absence and at low concentrations of exogenous Dol-*P*.

Effect of UDP-GlcNAc concentration on the formation of lipid-linked GlcNAc glycosides

Figure 3 shows how initial rate of GlcNAc transfer to lipid acceptors depended on the concentration of UDP-GlcNAc substrate in the presence and in the absence of 50 μM GTP. GTP pretreatment produced a stimulation of GlcNAc incorporation in microsomes at all UDP-GlcNAc concentrations. Calculation of kinetic parameters based on these data is precluded because the observed process involved the simultaneous action of two enzymes, UDP-GlcNAc:Dol-*PP*-GlcNAc *N*-acetylglucosaminyltransferase (*N*-acetylglucosaminyltransferase) and UDP-GlcNAc:Dol-*P* *N*-acetylglucosaminyl 1-phosphotransferase (*N*-acetylglucosaminyl 1-phosphotransferase). Both the mono- and the di-glycosidic lipids were formed from Dol-*P* and UDP-[^{3}H]GlcNAc during the course of the assay (see below).

Selectivity of GTP-stimulation toward *N*-acetylglucosaminyltransferase and *N*-acetylglucosaminyl 1-phosphotransferase

Figure 4 depicts the t.l.c. separation of the two lipid-linked GlcNAc-containing reaction products formed after 6 min of incubation. T.l.c. analysis of the chloroform/methanol extract revealed that Dol-*PP*-GlcNAc, which contained 80 ± 2% ($n = 7$) of total Dol-*P* sugar-associated radiolabel, was the predominant reaction product formed by microsomes in the absence of GTP. In the presence of GTP, the formation of Dol-*PP*-$(GlcNAc)_2$, which contained 65 ± 2% ($n = 7$) of the total Dol-*P* sugar-associated radiolabel, was significantly increased ($P < 0.0001$). Two sets of experiments were performed to address the question whether one of the two steps required for Dol-*PP*-$(GlcNAc)_2$ formation was selectively activated. First, we considered the possibility that a pool of preformed Dol-*PP*-GlcNAc was present in the microsomes which became involved in formation of Dol-*PP*-$(GlcNAc)_2$ upon GTP activation. This possibility was ruled

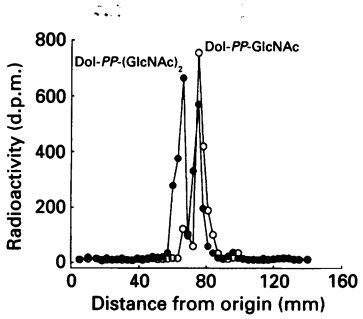

Figure 4 T.l.c. of GlcNAc-containing labelled dolichol derivatives

Microsomes were preincubated for 30 min at 37 °C in the presence (●) or absence (○) of 50 μM GTP. The concentration of added, exogenous Dol-*P* was 80 μg/ml. The enzyme assay was started by addition of 14 μM UDP-[^{3}H]GlcNAc, and incubation was continued for 6 min. T.l.c. of the chloroform/methanol extract was done as outlined in the Materials and methods section. The ordinate represents the amount of radiolabel recovered in each of the 3 mm lanes. The data shown are from one representative out of seven experiments.

out by our finding that no *N*-acetylglucosamine was incorporated when the microsomes were incubated with GTP and 2.5 μg/ml tunicamycin, which is a selective inhibitor of *N*-acetylglucosaminyl 1-phosphotransferase (Schwartz and Datema, 1980; Godelaine and Beaufay, 1983). In the GTP-pretreated microsomes, GlcNAc transfer in the presence and absence of tunicamycin was, respectively, 1.1 ± 0.8 and 71 ± 12 pmol of *N*-acetylglucosamine/min per mg protein (both $n = 4$). This provided evidence that the increased formation of Dol-*PP*-$(GlcNAc)_2$ in the GTP-pretreated microsomes could not solely depend on stimulation of the second, *N*-acetylglucosaminyltransferase-catalysed, step. Secondly, we examined whether GTP either equally affected both steps required for Dol-*PP*-$(GlcNAc)_2$ formation or preferentially stimulated the first, *N*-acetylglucosaminyl 1-phosphotransferase-catalysed, step. Microsomes were incubated for 30 min at 37 °C with UDP-[^{3}H]GlcNAc in the absence of both GTP and the ATP-regenerating system (Table 2); 2 mM NAD^+ was added to protect the sugar nucleotide. The predominant reaction product formed during this initial incubation was Dol-*PP*-GlcNAc (condition A). Following the first 30 min incubation period, tunicamycin without GTP (condition B) or with GTP (condition

Table 2 Conversion of Dol-*PP*-GlcNAc into Dol-*PP*-$(GlcNAc)_2$ in the presence of GTP and tunicamycin

In all five conditions, native microsomes were first incubated for 30 min at 37 °C in incubation medium devoid of an ATP-regenerating system, but containing 2 mM NAD^+ and 14 μM UDP-[^{3}H]GlcNAc. Five conditions were tested in parallel. In condition A the reaction products were separated by t.l.c. after this initial 30 min incubation period. In the four other conditions an ATP-regenerating system was added and the incubation at 37 °C was continued for another 39 min in the presence of 2.5 μg of tunicamycin/ml (B), in the presence of 2.5 μg tunicamycin/ml and 50 μM GTP (C), in the absence of tunicamycin (D) and in the absence of tunicamycin, but the presence of 50 μM GTP (E). The lipid-linked derivatives were then isolated and separated by t.l.c. as described in the Materials and methods section. Each value represents the mean ± S.E.M. for four microsomal preparations.

Condition	GlcNAc incorporated (%) into: Dol-*PP*-GlcNAc	Dol-*PP*-$(GlcNAc)_2$	Lipid-linked GlcNAc (pmol/mg of protein)
A	79 ± 4	21 ± 4	167 ± 15
B	79 ± 3	21 ± 3	145 ± 14
C	69 ± 3	**31 ± 3**	152 ± 19
D	83 ± 3	17 ± 3	197 ± 14
E	57 ± 4	43 ± 4	338 ± 33

Table 3 Effect of GTP on the microsomal incorporation of GlcNAc into lipid and protein catalysed by microsomes permeabilized by *Staph. aureus* α-toxin

Native microsomes or microsomes permeabilized with *Staph. aureus* α-toxin (see the accompanying paper, Bossuyt and Blanckaert, 1993) were preincubated at 37 °C with 15 μM UDP-Glc, 15 μM GDP-Man, 2.5 mg/ml α-lactalbumin, 80 μg/ml Dol-*P* and the other reagents of the standard incubation medium (see the Materials and methods section) in the presence or absence of 50 μM GTP. After 30 min, UDP-[^{3}H]GlcNAc was added and incubation was continued for another 60 min. Incorporation of label into lipid (chloroform/methanol extract) and protein (trichloroacetic acid-precipitable) are expressed as pmol/mg protein. Values are means ± S.E.M. of seven determinations in four different microsomal preparations. When indicated by an asterisk, the mean is statistically significantly different ($P < 0.005$) from mean of the control condition without GTP.

	GlcNAc incorporated (pmol/mg of protein): Into lipid	Into protein
Untreated microsomes		
−GTP	196 ± 17	268 ± 17
+GTP	684 ± 39*	1460 ± 159*
α-Toxin-treated microsomes		
−GTP	124 ± 15	205 ± 15
+GTP	400 ± 22*	997 ± 83*

C) was added, and incubation at 37 °C was continued for 39 min. If GTP was a selective activator of the second step, the preformed Dol-*PP*-GlcNAc would be used to form Dol-*PP*-$(GlcNAc)_2$. The addition of GTP, however, did result in only a minor increase in the formation of chloroform/methanol-extractable radiolabel, with a corresponding conversion of Dol-*PP*-GlcNAc to Dol-*PP*-$(GlcNAc)_2$. Therefore, we conclude that GTP did not exert its effect on the *N*-acetylglucosaminyltransferase-catalysed step. Microsomes to which GTP but no tunicamycin was added after the initial incubation for 30 min (condition E), incorporated more GlcNAc ($P < 0.01$) as compared with microsomes to which neither GTP nor tunicamycin had been added. T.l.c. analysis of the reaction products revealed an increase ($P < 0.005$) in the formation of Dol-*PP*-$(GlcNAc)_2$ as compared with control microsomes to which neither GTP nor tunicamycin had been added (condition D). This provided verification that GTP was still able to bring about an effect in microsomes that had been preincubated for 30 min.

Collectively, our findings demonstrate that stimulation of *N*-glycosylation by GTP is critically dependent on stimulation of the first step, i.e. enhanced transfer of GlcNAc-1-P from UDPGlcNAc to Dol-P.

GTP stimulates the pathway for protein glycosylation

Dol-*PP*-GlcNAc is the metabolic precursor of the more complex glycosylated derivatives of dolichyl pyrophosphate and constitutes an obligatory intermediate in the transfer of core sugar residues to protein, which is the final event in the dolichol pathway. In a reaction mixture supplemented with exogenous Dol-*P* (80 μg/ml), all nucleotide-sugar donor substrates for the complete glycosylation pathway (14 μM UDP-[^{3}H]GlcNAc, 15 μM GDP-Man, and 15 μM UDP-Glc), and an acceptor substrate (2.5 mg of α-lactalbumin/ml), *N*-acetylglucosamine incorporation into trichloroacetic acid-precipitable material was enhanced by GTP-pretreatment of the microsomes. Following incubation for 60 min at 37 °C, we found 268 ± 17 pmol of GlcNAc incorporated into protein/mg of microsomal protein ($n = 7$) in control microsomes, without GTP, and 1460 ± 159 pmol of GlcNAc incorporated into protein/mg of protein ($n = 7$) in the GTP-pretreated microsomes.

GTP-stimulation of the pathway for protein glycosylation is not caused by an altered ER membrane permeability

The mechanism by which GTP exerts an influence on *N*-glycosylation is unknown. Godelaine et al. (1983) reported that the structure-linked latency of mannose-6-phosphatase was reduced on incubation of stripped rough-ER-derived microsomes with GTP. As similar conditions enhanced the dolichol-mediated glycosylation of protein in microsomes, Godelaine et al. (1983) suggested that GTP might activate dolichol-mediated glycosylation by lowering the permeability barrier for nucleotide-sugars. Since in those studies control microsomes displayed relatively poor latency of mannose-6-phosphatase (< 90 %), we reinvestigated the effect of GTP on membrane permeability using microsomal preparations which were well sealed (> 95 % latency of mannose-6-phosphatase) in their native state. Mannose-6-phosphatase activity, assayed under the conditions of our glycosyltransferase assay, was 0.85 ± 0.10 ($n = 4$) and 1.92 ± 0.12 nmol/min per mg protein ($n = 4$) in, respectively, control microsomes and GTP-pretreated (preincubation for 30 min at 37 °C in the presence of 50 μM GTP) microsomes. The corresponding total phosphatase activity, measured in *Staph. aureus*-α-toxin-permeabilized microsomes, was 11.4 ± 1.1 nmol/min per mg protein ($n = 4$). These data confirm Godelaine et al.'s (1983) observation that GTP slightly decreases mannose-6-phosphatase latency, which can be interpreted as indicating that permeability of the microsomal membrane is somewhat enhanced in native microsomes.

However, the idea that a change in microsomal-membrane-permeability properties induced by GTP might account for the enhancement of protein glycosylation is no longer tenable. First, recent evidence suggests that the reactions responsible for GlcNAc transfer to Dol-*P* rely on catalytic centres that are exposed at the cytoplasmic side of the ER [Abeijon and Hirschberg, 1990; Kean, 1991; the accompanying paper (Bossuyt and Blanckaert, 1993)]. Second, the results, shown in Table 3, demonstrate that GTP maintained its stimulatory effect on the pathway for *N*-glycosylation in fully permeabilized microsomes. In α-toxin-treated microsomes, in which the nucleotide-sugar

donor substrate had unlimited access to the lumen of the microsomes, the GTP stimulation factor of GlcNAc incorporation in lipid and protein was, respectively, 3.2 (range 2.5–4.6; $n = 7$) and 4.9 (range 4.8–5.9; $n = 7$). This was comparable with the GTP stimulation factor in native microsomes, which was, respectively, 3.4 (range 2.8–4.1; $n = 7$) and 5.4 (range 4.3–7.7; $n = 7$). Hence these observations are inconsistent with the notion that the GTP effect is caused by an altered membrane permeability for nucleotide-sugars.

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