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Site of stimulation by mannosyl-P-dolichol of GlcNAc-lipid formation by microsomes of embryonic chick retina

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Mannosyl-P-dolichol (man-P-dol) has been shown to stimulate the early reactions of the dolichol pathway, specifically, the biosynthesis of GlcNAc-P-P-dol and GlcNAc-GlcNAc-P-P-dol, and thus may play a regulatory role in glycoprotein biosynthesis. The site of action of man-P-dol has previously been suggested to be the GlcNAc-transferase concerned with the formation of the monoglucosaminyl derivative. Since the concentration of the chitobiosyl compound also increases as a result of the presence of man-P-dol, the immediate site of the activation was reexamined. The effect of man-P-dol on the formation of GlcNAc-GlcNAc-P-P-dol using GlcNAc-P-P-dol synthesized *in situ* or added exogenously as the substrate was investigated. In addition, the distribution of radioactivity in the glucosaminyl constituents of the products under the stimulatory conditions was determined. The results of these studies supported the conclusion that the stimulation of GlcNAc-lipid synthesis by man-P-dol is due to the enhanced synthesis of GlcNAc-P-P-dol. It is not a result of the activation of the GlcNAc-transferase catalyzing the attachment of the second GlcNAc residue for the biosynthesis of the chitobiosyl derivative.

Keywords: mannosyl-P-dolichol, GlcNAc-P-P-dolichol, GlcNAc-GlcNAc-P-P-dolichol, site of stimulation, retina

Abbreviations: GlcNAc-P-P-dol, *N*-acetylglucosaminylpyrophosphoryldolichol; GlcNAc-GlcNAc-P-P-dol, *N*-acetylglucosaminyl-*N*-acetylglucosaminylpyrophosphoryldolichol; chito, *N*-*N*′-diacetylchitobiose; man-P-dol, mannosylphosphoryldolichol; TX-100, triton X-100; Tes, 2-{[*tris*-(hydroxymethyl)-methyl]-amino}-ethanesulfonic acid.

Introduction

Previous studies have demonstrated that mannosyl-P-dolichol (man-P-dol), in addition to functioning as a donor of mannose residues in biosynthetic reactions, also acts as an activator of the GlcNAc-transferases that catalyse the biosynthesis of the initial reactions of the dolichol pathway, i.e. the formation of GlcNAc-P-P-dol and GlcNAc-GlcNAc-P-P-dol [1–6]. Extensive stimulation of their biosynthesis by man-P-dol has been observed. Evidence has been presented [2] suggesting that the most likely target of the stimulation is UDP-GlcNAc:dolichyl phosphate *N*-acetylglucosamine-1-phosphate transferase (GPT1), the enzyme which catalyses the formation of the first intermediate of the dolichol pathway, GlcNAc-P-P-dol. However, the synthesis of the next intermediate in the pathway, GlcNAc-GlcNAc-P-P-dol, which utilizes the mono-GlcNAc compound as its immediate precursor in a sequential reaction, also increases in the presence of man-P-dol. The stimulatory phenomenon thus could be directed at both reactions. In order to obtain additional evidence concerning the immediate site of the stimulatory effect, two types of experiments were performed. The first was to examine the influence of the activating compound on the synthesis of GlcNAc-GlcNAc-P-P-dol using GlcNAc-P-P-dol synthesized *in situ* as the substrate and also using the chemically synthesized compound added exogenously. The second was to determine the distribution of radioactivity in the GlcNAc residues of the products that resulted from the stimulation.

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Materials and methods

Enzyme preparations

Microsomes from the retina of the 15-day embryonic chick, used as the enzyme source for the GlcNAc-transferases, were prepared as described previously [1, 2] and stored at −70 °C until used.

Materials

Man-P-dol was synthesized using an extract from *Micrococcus leuteus* as enzyme source, obtained from Drs C. Waechter and J. Rush of the University of Kentucky School of Medicine, as described previously [7]. GlcNAc-P-P-dol, synthesized chemically [8], was provided by Dr Barbara Imperiali of the California Institute of Technology. The following materials were obtained from the indicated sources: UDP-[6-^{3}H]GlcNAc (30 Ci mmol^{-1}), and GDP-[^{14}C]mannose (321 mCi mmol^{-1}), from DuPont-New England Nuclear Corp. (Boston, MA); [6-^{3}H]glucosamine (30 Ci mmol^{-1}), and EcoLume Scintillation cocktail, from ICN (Costa Mesa, CA); dolichol phosphate, Grade III, tunicamycin and sodium borohydride, from Sigma Chemical Co., (St Louis, MO). [^{3}H]Glucosaminitol was prepared from [^{3}H]glucosamine by reduction with sodium borohydride. All other reagents and chemicals were the highest grade commercially available.

GlcNAc-lipid synthesis

Suspensions of microsomes were incubated at 37 °C in a mixture containing dolichol phosphate or GlcNAc-P-P-dol, non-radioactive UDP-GlcNAc or UDP[^{3}H]GlcNAc (see legends to the Tables), Tes buffer (0.2 M, pH 7.4), $MgCl_2$ (27 mM), TX-100 (0.15%), (basal conditions), or under stimulatory conditions in the presence of man-P-dol as described previously [1–3]. The reactions were stopped by the addition of 20-fold volumes of chloroform:methanol (2:1, by vol), and the mixture extracted by the procedure of Folch *et al.* [9]. The washed lower phase resulting from this procedure was evaporated to dryness and the radioactivity determined.

Other analytical procedures

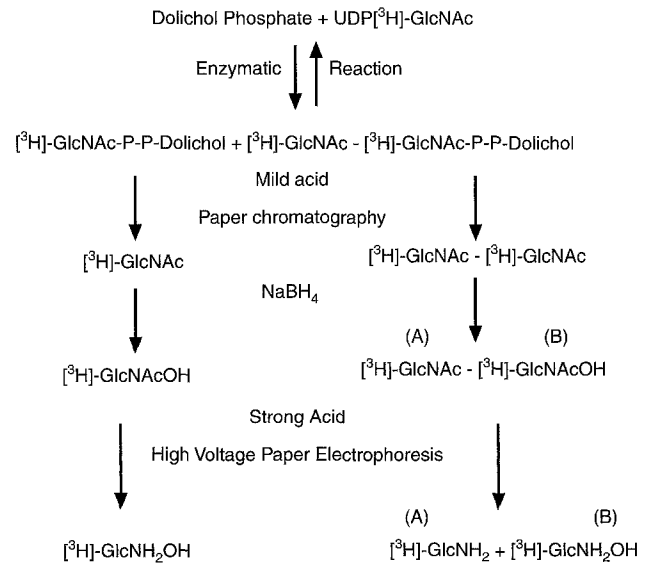
Paper chromatography was carried out on Whatman No. 1 paper by descending technique for 15 h, at room temperature using 1-butanol:pyridine:water (6:4:3, by vol) as solvent system. High voltage paper electrophoresis was carried out on a Gilson High Voltage Electrophorator, model D on Whatman 3MM paper in 1% sodium tetraborate, pH 9.4 for 60 min at 53 V cm^{-1}. The chromatograms and electrophoretograms were dried, cut into 1 × 4 cm zones and analysed for radioactivity by scintillation spectrometry in the presence of 0.5 ml of water and 5 ml of EcoLume. The migrations on the papers of standard GlcNAc and *N,N′*-diacetylchitobiose were detected by the aniline/diphenylamine reaction [10]. The migrations of standard [^{3}H]glucosaminitol and standard [^{3}H]glucosamine after electrophoresis were determined by scintillation spectrometry. Mild acid hydrolysis was carried out at 50 °C for 100 min in 0.1 N HCl-tetrahydrofuran [11]. Strong acid hydrolysis was performed in 4 N HCl for 6 h at 100 °C. Reduction was carried out with 0.25 M $NaBH_4$ in 0.025 N NaOH overnight at room temperature. Protein was measured by the method of Lowry *et al.* [12].

Distribution of radioactivity in GlcNAc residues of the products

Incubations were carried out in the presence of man-P-dol, as described above. The distribution of radioactivity in the products was analysed in the same manner as described previously for the distribution of label under basal conditions [13]. In short, this involved the following steps carried out sequentially: mild acid hydrolysis of the GlcNAc-lipids present in the lower phase from the Folch-washing; mixed bed ion exchange chromatography of the cleaved products; separation of GlcNAc and GlcNAc-GlcNAc by paper chromatography and recovery of these materials from the chromatogram; reduction of each with $NaBH_4$; mixed bed ion exchange chromatography; evaporation in the presence of methanol; strong acid hydrolysis; high voltage paper electrophoresis in borate buffer; measurement of the radioactivity of the products on the electrophoretogram. A flow diagram is presented outlining these steps (Scheme 1).

Rationale and design of experiments

As shown in these and in previous studies [1, 2] the major products extractable into chloroform:methanol (2:1) that are formed by embryonic chick retina microsomal



Scheme 1.

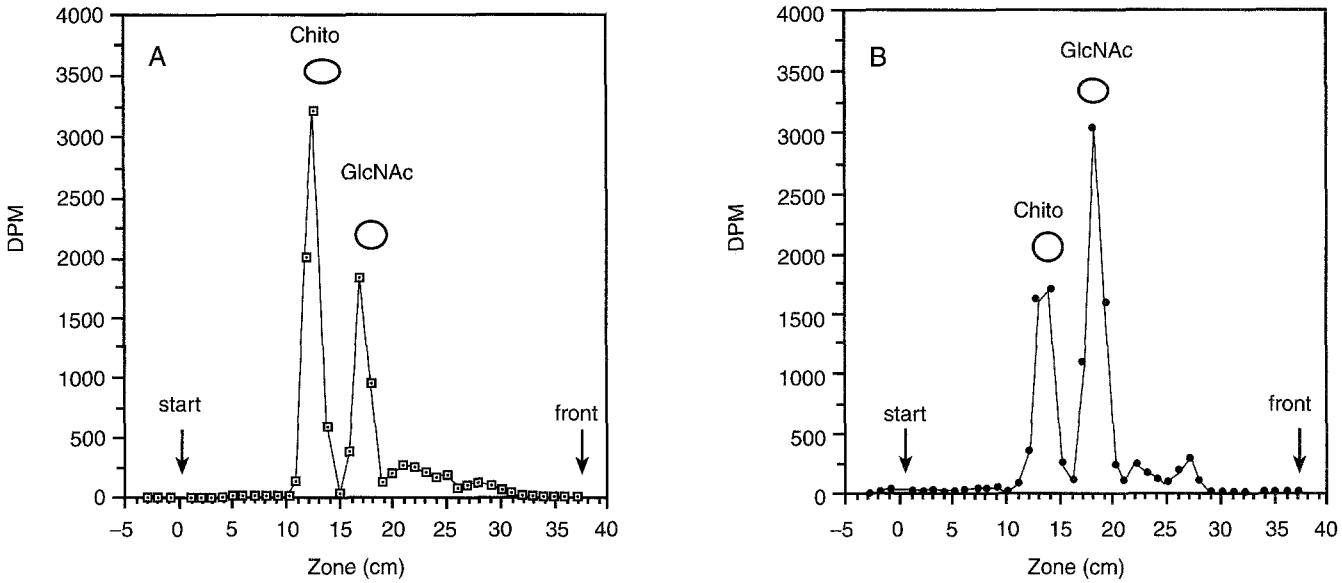


Figure 1. Paper chromatography of saccharides liberated by mild acid hydrolysis of GlcNAc-lipids formed under basal conditions (A), or stimulatory conditions (B) in the presence of man-P-dol (6.7 μM).

membranes when incubated under basal conditions or in the presence of man-P-dol, are GlcNAc-P-P-dol and GlcNAc-GlcNAc-P-P-dol. Under stimulatory conditions, however, the amounts of these materials are greatly increased and also, as seen in Fig. 1, their relative proportions are changed. In order to explore the site of the effects brought about by man-P-dol, a two-phase incubation protocol was followed. In the first phase, embryonic chick retina microsomes were incubated for 10 min in the presence of a complete GlcNAc-lipid synthesizing system containing dolichol phosphate, detergent, buffer and metal ions as described above, and using non-radioactive UDP-GlcNAc as substrate. Under these conditions unlabelled GlcNAc-P-P-dol and unlabelled GlcNAc-GlcNAc-P-P-dol would be formed. During the second phase UDP-[^{3}H]GlcNAc was added and the incubation continued for an additional 10 min in the presence or absence of the activating compound, man-P-dol, added as a detergent micelle, and in the presence or absence of tunicamycin. In the presence of the antibiotic, [^{3}H]GlcNAc-GlcNAc-P-P-dol would be the only radioactive glucosaminyl derivative formed under basal or stimulating conditions, resulting from the addition of [^{3}H]GlcNAc to non-radioactive GlcNAc-P-P-dol that had been formed during the first phase. In the absence of tunicamycin, [^{3}H] GlcNAc-P-P-dol and tritiated GlcNAc-GlcNAc-P-P-dol would be produced, the latter being a mixture of molecules with one and two GlcNAc residues labelled. By comparison with appropriate controls, the contributions of each component and the effect of man-P-dol on their synthesis can be calculated. A detailed description of this experiment is provided in the legend to Table 1.

Table 1. Labelling of [^{3}H]GlcNAc-lipids in the presence and absence of tunicamycin and man-P-dolichol

	Condition	*GlcNAc-lipid (pmol)*	*Chito-lipid (pmol)*	*Total dpm*
a	Basal; control	2.3	1.8	1530
b	Basal + tunicamycin during phase 2	0	0.91	480
c	Stimulated (man-P-dol during phase 2)	70	22	30100
d	Stimulated (man-P-dol + tunicamycin during phase 2)	0	0.77	409

In all conditions during the first phase incubation (10 min, 37 °C) there were present, dolichol phosphate (24 μM), non-radioactive UDP-GlcNAc (51.0 μM), Tes buffer, TX-100, $MgCl_2$, and enzyme (retina microsomes, 0.18 mg protein), as described in Materials and methods in a total volume of 0.12 ml. During the second phase incubation (10 min at 37 °C), 1.56 nmol of UDP-[^{3}H]GlcNAc was added (51.2 μM final concentration, final specific activity in the system, 265 dpm pmol^{-1}), and the following components: condition (a), 10 μl DMSO, 10 μl 0.75% TX-100; condition (b) tunicamycin (3.0 μg in 10 μl DMSO), 10 μl 0.75% TX-100; condition (c), 10 μl DMSO, man-P-dol (1.0 nmol dispersed in 10 μl 0.75% TX-100); condition (d) tunicamycin (3.0 μg in 10 μl DMSO), man-P-dol (1.0 nmol dispersed in 10 μl 0.75% TX-100) in a total volume of 0.15 ml. The reactions were stopped by the addition of C:M (2:1), and the radioactivity determined in the chloroform-rich phase after solvent partitioning as described in Materials and methods.

The calculations of the labelled products were based on the following considerations: The ratio of GlcNAc/GlcNAc-GlcNAc of 0.65 under basal conditions and 1.6 under stimulatory conditions (presence of man-P-dol) as determined in preliminary experiments was used in calculating the distribution of label in GlcNAc-P-P-dol and GlcNAc-GlcNAc-P-P-dol under each condition. From the specific activity of UDP-[^{3}H]GlcNAc used as the substrate and the specific activity of chitobiose (twice that of GlcNAc), the molar amounts of GlcNAc-lipids were derived. The same results were obtained in calculations based on evaluating the individual contributions to chitobiose with one and two GlcNAc residues labelled as described in the text.

The effect of man-P-dol on the synthesis of GlcNAc-GlcNAc-P-P-dol using authentic GlcNAc-P-P-dol as substrate was also examined.

Results and discussion

Labelling from GlcNAc-P-P-dol synthesized in situ

Shown in Table 1 are the results of experiments using GlcNAc-P-P-dol synthesized *in situ* as the substrate. Controls demonstrated that GlcNAc-lipid synthesis was linear over the entire time course of the experiment (first and second phases), and also that tunicamycin acted in the manner expected by inhibiting the biosynthesis of GlcNAc-P-P-dol [14] (see Table 2). The first phase of the incubation, carried out using unlabelled UDP-GlcNAc, produced products whose further conversion during the second phase was performed in the presence of the radioactive sugar nucleotide, i.e. only during the second phase does labelling occur. Under basal conditions (absence of man-P-dol) the labelling which occurred in the presence of tunicamycin added in the second phase (line b), would be due only to the addition of [^{3}H]GlcNAc to GlcNAc-P-P-dol preformed during the first phase producing the singly labelled chitobiosyl derivative, [^{3}H]GlcNAc-GlcNAc-P-P-dol. In the absence of tunicamycin under basal or stimulatory conditions, the chitobiosyl derivative would be a mixture of molecules with one and two GlcNAc residues labelled. As seen in Table 1, in the presence of man-P-dol, total GlcNAc-lipid synthesis was stimulated about 20-fold (line c) over the basal level (line a), and was composed of a 30-fold increase in the concentration of GlcNAc-P-P-dol and a 13-fold increase in GlcNAc-GlcNAc-P-P-dol. However, in the presence of both tunicamycin and man-P-dol (line d) no increase in labelling or the concentration of the chitobiosyl derivative was obtained, the labelling being similar to that obtained under basal conditions in the presence of tunicamycin (line b). It is thus clear that the increase over the basal concentration of GlcNAc-GlcNAc-P-P-dol (13-fold, line c) which also occurred in the presence of man-P-dol was due only to the increased concentration of GlcNAc-P-P-dol formed by the presence of the activating compound during the second phase and now available for use as a substrate for chito formation. Thus, the stimulation of GlcNAc-lipid synthesis brought about by man-P-dol is due directly to its effect on GPT1 and not to the activation of the GlcNAc-transferase catalyzing the attachment of the second GlcNAc residue for the biosynthesis of the chitobiosyl derivative.

Table 2. Use of chemically synthesized GlcNAc-P-P-dolichol as substrate

	Substrate	*Incubation Conditions*	*dpm[^{3}H]*
1	dolichol phosphate	Basal	3760
2	"	+[^{14}C]-man-P-dol	22850
3	"	+[^{14}C]-man-P-dol + tunicamycin	59
4	GlcNAc-P-P-dol	Basal	8640
5	"	+[^{14}C]-man-P-dol + tunicamycin	7910
6	"	+ tunicamycin	8640

Incubations were carried out for 10 min at 37 °C in the presence of UDP[^{3}H]GlcNAc (50 μM, 135 × 10^6 dpm μmol^{-1}), Tes buffer (0.2 M, pH 7.4), TX-100 (0.15%), $MgCl_2$ (27 mM), 6.7% DMSO and enzyme (0.26 mg protein) in a total volume of 0.15 ml. Dolichol phosphate (15.6 μM) was added to tubes 1–3, and GlcNAc-P-P-dol (0.2 mg ml^{-1}) was added to tubes 4–6. Tunicamycin (20 μg ml^{-1}) and [^{14}C]-man-P-dol (1.15 × 10^5 dpm μmol^{-1}, 22 μM) were present where indicated. The reactions were stopped by the addition of chloroform:methanol (2:1, by vol). After partitioning by the Folch procedure [9], the radioactivity in the washed organic phase was determined. Established techniques for double label analysis were employed.

Using exogenously added GlcNAc-P-P-dol

As can be seen in Table 2, under conditions where man-P-dol brought about a six-fold stimulation in GlcNAc-lipid synthesis (line 2) compared to basal conditions (line 1), the presence of man-P-dol did not result in an enhancement in labelling over the new basal level using authentic GlcNAc-P-P-dol as substrate (lines 4 and 5). The increased labelling at the new basal level in the presence of GlcNAc-P-P-dol was due to its conversion to the chitobiosyl derivative. Thus, after mild acid hydrolysis of the [^{3}H]-labelled product formed under these conditions, a single radioactive component was detected by paper chromatography that migrated with standard *N-N′* diacetylchitobiose (data not shown). In the experiments using GlcNAc-P-P-dol as substrate in the presence of man-P-dol, tunicamycin was added (line 5) to block the synthesis of [^{3}H]GlcNAc-P-P-dol which would have been formed from dolichol-phosphate present as a breakdown product of man-P-dol due to the latter's instability, and thus mask its lack of effect (data not shown). While tunicamycin completely blocked GlcNAc-lipid synthesis under basal (data not shown) or stimulatory conditions (line 3), this antibiotic had no effect on the addition of a second GlcNAc residue to GlcNAc-P-P-dol (line 6).

The results of this experiment showing a lack of stimulation by man-P-dol on exogenously added GlcNAc-P-P-dol are the same as the experiment described above in which this substrate was synthesized *in situ*.

Distribution of label in GlcNAc-lipids synthesized in the presence of man-P-dolichol

As seen in Fig. 1, paper chromatography effectively separates GlcNAc and *N-N′* diacetylchitobiose (chito) which had been cleaved by mild acid hydrolysis from their respective dolichol pyrophosphoryl derivatives. GlcNAc-GlcNAc-P-P-dolichol is the major product formed under basal conditions (Fig. 1A), and GlcNAc-P-P-dol, the major product in the presence of man-P-dol (Fig. 1B), as described previously [7, 15].

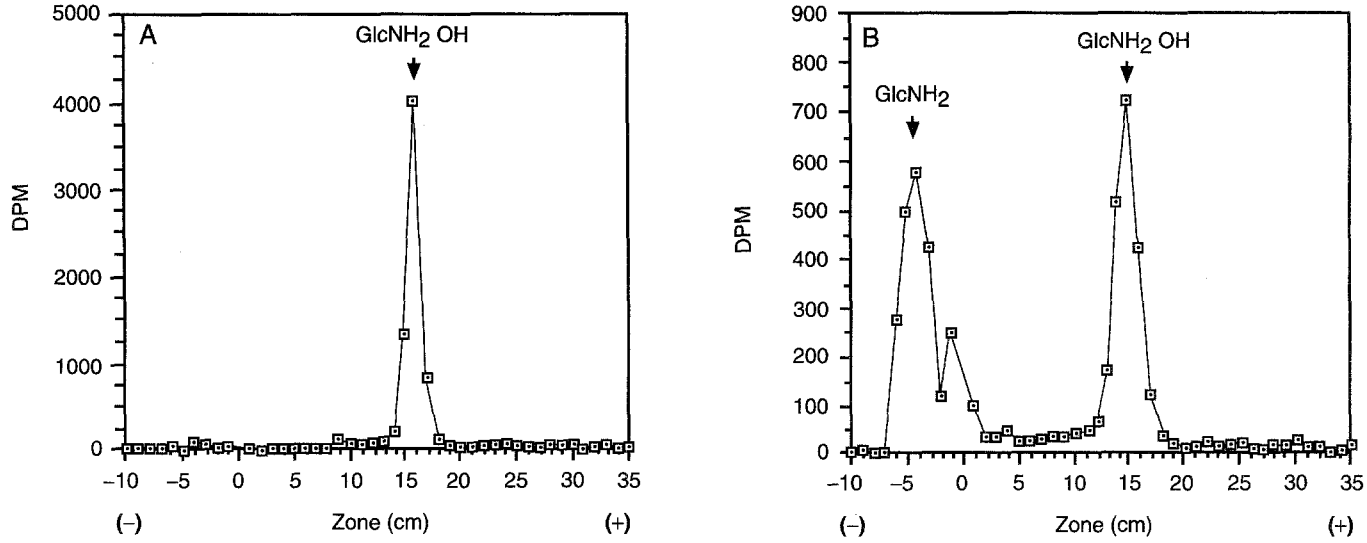


Figure 2. Analysis by high voltage paper electrophoresis of the glucosaminyl components of the GlcNAc-lipids synthesized in the presence of mannosyl-P-dolichol. The arrows indicate the migrations of standard [^{3}H]glucosamine and standard [^{3}H]glucosaminitol. A. The product derived from GlcNAc-P-P-dol. B. The products derived from GlcNAc-GlcNAc-P-P-dol.

(The ratios of the products produced under basal and stimulated conditions are used in the calculations described in the legend to Table 1.) Reduction with $NaBH_4$ of chito recovered from the paper chromatogram, formed *N-N'*diacetylchitobiitol. Strong acid hydrolysis cleaved and N-deacetylated the latter producing [^{3}H]glucosamine and [^{3}H]glucosaminitol which were separated by high voltage paper electrophoresis in borate buffer (Fig. 2). (An outline of these steps is presented in Scheme 1.) In this manner the extent of labelling of the individual amino sugar components of chito was determined. When these same procedures were applied to GlcNAc-P-P-dol, a single radioactive component was obtained that migrated with the expected product, glucosaminitol. Data from an experiment of this type is summarized in Table 3. About 2.5-fold greater labelling of the GlcNAc residue from GlcNAc-P-P-dol was obtained compared to each of the amino sugar residues of the chitobiosyl derivative, consistent with the stimulatory action being the formation of GlcNAc-P-P-dol under these experimental conditions. The similarity in labelling of each of the glucosaminyl residues of the chitobiosyl derivative indicates an approach to isotopic equilibrium obtained under the conditions used in these experiments, similar to previous studies examining the distribution of label under basal conditions with rat liver microsomes [13]. In addition, the stoichiometry of the labelling of the monoGlcNAc and chitobiosyl derivatives obtained by the derivitization and isolation procedures is similar to that obtained by the direct paper chromatographic analysis of the products.

While reversibility of the formation of GlcNAc-P-P-dol has been demonstrated [16], little is known of the kinetics of this process. Reversibility of this reaction under basal or stimulated conditions in the absence of tunicamycin would result in the formation of UDP[^{3}H]GlcNAc and a decrease in the measured amount of [^{3}H]GlcNAc-P-P-dol. The specific activity of the sugar nucleotide used in the calculations would be unaffected by this process. The isolation procedures would remove the sugar nucleotide formed by the reversal. The labelled GlcNAc-P-P-dol which was obtained by the procedures

Table 3. Distribution of [^{3}H] in products after stimulation by mannosyl-P-dolichol

Products	*Components Assayed*	*dpm*	*Ratio*
[^{3}H]GlcNAc-[^{3}H]GlcNAc-P-P-dol	[^{3}H]$GlcNH_2$	2740	1.00
	[^{3}H]$GlcNH_2$-OH	2480	0.91
[^{3}H]GlcNAc-P-P-dol	[^{3}H]$GlcNH_2$-OH	6530	2.4

GlcNAc-lipid synthesis was carried out for 10 min at 37 °C in the presence of dolichol phosphate, man-P-dol, UDP[^{3}H]GlcNAc, enzyme, Mg^{2+}, and TX-100, as described in Materials and methods. After isolation by solvent partitioning, the products were treated as described in Scheme 1. The radioactivity of the final derivatives after separation by high voltage paper electrophoresis in borate buffer was determined by scintillation spectrometry.

used here would therefore be the net amount from the forward and reverse reactions, and the stimulated formation of this compound by man-P-dol would be even greater than that actually calculated. While not examined in detail, the formation of GlcNAc-GlcNAc-P-P-dol does not appear to be reversible. The participation of other components of the dolichol pathway would not be involved in these considerations since little or no labelling of oligosaccharide-lipids or the glycoprotein-containing residue by the retina microsomes occurs under these conditions as shown previously [17].

Thus, the results from the experimental approaches used in the present studies; (1) directly measuring the effect of man-P-dol on product formation using as substrate GlcNAc-P-P-dol synthesized *in situ* or added exogenously, and (2) chemically measuring the distribution of radioactivity in the products of the reaction; support the conclusion that the immediate site of the stimulation of GlcNAc-lipid synthesis by man-P-dol is UDP-GlcNAc:dolichyl phosphate *N*-acetylglucosamine-1-phosphate transferase, the enzyme which catalyzes the formation of GlcNAc-P-P-dol, and not the GlcNAc-transferase catalysing the attachment of the second GlcNAc residue.

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References

1. Kean EL (1982) *J Biol Chem* **257**: 7952–54.
2. Kean EL (1985) *J Biol Chem* **260**: 12561–71.
3. Kean EL, DeBrakeleer DJ (1986) *Arch Biochem Biophys* **250**: 146–52.
4. Kaushal GP, Elbein AD (1985) *J Biol Chem* **260**: 16303–9.
5. Shailubhai K, Dong-Yu B, Saxena ES, Vijay IK (1988) *J Biol Chem* **263**: 15964–72.
6. Carson DD, Farrar JD, Laidlaw J, Wright DA (1990) *J Biol Chem* **265**: 2947–55.
7. Kean EL, Rush JS, Waechter CJ (1994) *Biochemistry* **33**: 10508–12.
8. Imperiali B, Zimmerman JW (1990) *Tetrahedron Lett* **31**: 6485–88.
9. Folch J, Lees M, Sloane Stanley GH (1957) *J Biol Chem* **226**: 497–509.
10. Block RJ, Durrum EL, Zweig G (1958) In *Paper Chromatography and Paper Electrophoresis*, second edition p. 183. NY: Academic Press.
11. Lucas JJ, Waechter CJ, Lennarz WJ (1975) *J Biol Chem* **250**: 1992–2002.
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) *J Biol Chem* **193**: 265–75.
13. Kean EL (1992) *Biochem Cell Biol* **70**: 413–21.
14. Tkacz JS, Lampen JO (1975) *Biochem Biophys Res Commun* **65**: 248–57.
15. Kean EL (1991) *J Biol Chem* **266**: 942–46.
16. Harford JB, Waechter CJ (1979) *Arch Biochem Biophys* **197**: 424–35.
17. Kean EL (1980) *J Biol Chem* **255**: 1921–27.