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Enzymatic Utilization of P^1-Di-*N*-acetylchitobiosyl P^2-Dolichyl Pyrophosphate and Its Chemical Synthesis*

(glycoprotein biosynthesis/lipid intermediate/mannose-containing oligosaccharide/ di-*N*-acetylchitobiosyl phosphate)

JOSIAH F. WEDGWOOD†, CHRISTOPHER D. WARREN‡, ROGER W. JEANLOZ‡, AND JACK L. STROMINGER†

† The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138; and ‡ Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, Mass. 02114

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ABSTRACT **Fully acetylated chitobiose was treated with phosphoric acid to give a mixture of products from which 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α,β-D-glucopyranosyl phosphate (Ac_3GlcNAc-Ac_2GlcNAc-*P*) was isolated by preparative thin layer chromatography. Treatment of this compound with P^1-diphenyl P^2-dolichyl pyrophosphate gave an acetylated pyrophosphate diester, which was purified chromatographically and deacetylated. The product, P^1-di-*N*-acetylchitobiosyl P^2-dolichyl pyrophosphate (Dol-*P*-*P*-GlcNAc-GlcNAc), was readily separated from P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (Dol-*P*-*P*-GlcNAc) by thin layer chromatography in several solvent systems. Addition of this compound to human lymphocyte membrane preparations led to the formation of a mannose-containing derivative which appears to be an oligosaccharide phospholipid, as judged by its behavior on DEAE-cellulose chromatography and by its hydrolysis to give an oligosaccharide containing more than four monosaccharide units.**

Incubations of membrane preparations from a variety of animal sources with nucleoside diphosphate sugar compounds give glycosyl polyprenyl phosphates, which are intermediates in the biosynthesis of some glycoproteins (1–3). Dolichyl β-D-mannopyranosyl phosphate (Dol-*P*-Man) has been identified as a product of the incubation with GDP-Man in calf pancreas and in human lymphocytes (4). Dol-*P*-*P*-GlcNAc has been shown to be formed from UDP-GlcNAc in calf pancreas (5). Rat liver microsomes have been shown to synthesize lipid-linked pyrophosphate diesters of GlcNAc and di-*N*-acetylchitobiose (6). Lipid-bound oligosaccharide phosphates have also been isolated (3, 7, 8, 9). Similar compounds found in bacterial and plant systems are involved in the formation of other complex glycans (10).

It has been suggested that a mannose-containing oligosaccharide lipid is formed by the transfer of mannose from GDP-Man or Dol-*P*-Man to Dol-*P*-*P*-GlcNAc-GlcNAc (8). An authentic sample of Dol-*P*-*P*-GlcNAc-GlcNAc has been obtained by chemical synthesis. This compound stimulates the incorporation, in human lymphocyte membrane preparations, of mannose into a compound with the properties of an oligosaccharide phospholipid containing more than four monosaccharide units. A similar oligosaccharide phospholipid, which contains GlcNAc as well as mannose, is formed when Dol-*P*-*P*-GlcNAc is added.

Abbreviations: GlcNAc-*P*, 2-acetamido-2-deoxy-D-glucopyranosyl phosphate; Ac_3GlcNAc-*P*, 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-glucopyranosyl phosphate; Ac_3GlcNAc-Ac_2GlcNAc-*P* and GlcNAc-GlcNAc-*P*, peracetyl- and di-*N*-acetyl-α,β-chitobiosyl phosphates, respectively; Dol-*P*, dolichyl phosphate; Dol-*P*-Man, dolichyl β-D-mannopyranosyl phosphate; Dol-*P*-*P*, dolichyl pyrophosphate; Dol-*P*-*P*-GlcNAc, P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate; Dol-*P*-*P*-GlcNAc-GlcNAc, P^1-di-*N*-acetylchitobiosyl P^2-dolichyl pyrophosphate [P^1-2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α,β-D-glucopyranosyl P^2-dolichyl pyrophosphate].

* This is part IX of the series, "Lipid Intermediates of Complex Polysaccharide Biosynthesis." For part VIII, see ref. 14.

MATERIALS AND METHODS

Chemicals. GDP-[^{14}C]Man (160 mCi/mmol) and UDP-[^{3}H]GlcNAc (6.6 Ci/mmol) were obtained from New England Nuclear. Stachyose was from Pfanstiehl, and maltotriose from Pierce Chemicals. Dolichol was a gift of Dr. W. Jankowski, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa 12, Poland, or was obtained through the courtesy of Mr. D. Broida and Dr. T. Cori, Sigma Chemical Co., St. Louis, Mo. Other biochemicals were obtained from Sigma Chemical Co. Peracetylchitobiose was prepared as described by Spinola and Jeanloz (11). Dolichyl phosphate (Dol-*P*) was prepared as described (12). P^1-Diphenyl P^2-dolichyl pyrophosphate (13), Dol-*P*-*P*-GlcNAc (13), and dolichyl pyrophosphate (Dol-*P*-*P*) (14) were prepared according to Warren and Jeanloz.

General Methods. These were as described (15), except for the following solvent systems used for silica gel thin layer chromatography were: A, chloroform–methanol–water (60:25:4); B, chloroform–methanol–water (60:35:6); C, chloroform–methanol–water (10:10:3); D, 2,6-dimethyl-4-heptanone–acetic acid–water (20:15:2); E, 2-propanol–15 M ammonium hydroxide–water (6:3:1); and F, chloroform–methanol–15 M ammonium hydroxide–water (65:35:4:4). The solvent system used for cellulose thin layer chromatography was isobutyric acid–15 M ammonium hydroxide–water (66:1:33). Toluene and pyridine were dried over calcium hydride before use. DEAE-cellulose (DE-52, Whatman) was converted into the acetate form according to the directions of the manufacturer and then washed extensively with methanol to remove all water.

The proportion of GlcNAc was determined after hydrolysis at 105° in 4 M HCl for 2 hr with a Beckman Amino Acid Analyzer 120C, with di-*N*-acetylchitobiose as the standard. The proportion of acid-labile phosphate was determined by the method of Chen *et al.* (16) after digestion of the sample (50 μg) with concentrated H_2SO_4 at 150° for 1 hr.

Synthesis of 2-Acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α,β-D-glucopyranosyl Phosphate (GlcNAc-GlcNAc-P) (*Fig. 1, Structure* **2**). A solution of peracetyl chitobiose (0.24 g) in chloroform–tetrahydrofuran (1:1, 3 ml) was added to crystalline phosphoric acid (0.48 g). The mixture was stirred until a uniform suspension was obtained, and then the solvents were evaporated under a stream of N_2 gas. The residue was kept at 65° under reduced pressure for 3 hr, and the brown fused product was dissolved in tetrahydrofuran (10 ml). The stirred solution was cooled to −10°, neutralized with 0.15 M ammonium hydroxide (about 48 ml) to give pH 5–6, treated with pyridine (2 ml), and evaporated. Toluene was added and evaporated twice, and the solid residue was extracted with chloroform–methanol (1:1). After filtration and evaporation, the crude product (0.3 g) was dissolved in water (45 ml). The solution was extracted six times with chloroform (15 ml). Evaporation of the aqueous phase gave an amorphous product (0.24 g) that was purified by preparative thin layer chromatography. The chromatograms were developed three times in solvent B; they were dried in a stream of air between each development. The area containing the main product was located with a phosphate-specific spray; the material was extracted with solvent C by stirring overnight. Filtration through Celite, followed by evaporation, gave a residue, which was dissolved in chloroform–methanol (1:1). The solution was filtered through sintered glass and evaporated to yield amorphous $Ac_3GlcNAc$-$Ac_2GlcNAc$-*P* (Fig. 1, structure **1**; 55 mg), mp 163–164°, $[\alpha]_D^{25}$ −4° (c 1.05, methanol). The infrared spectrum showed peaks arising from $OCOCH_3$, GlcNAc, and PO_4. Calculated for $C_{26}H_{39}N_2O_{19}P \cdot 2H_2O$: C, 41.60; H, 5.78; N, 3.73; P, 4.13. Found: C, 41.19; H, 5.43; N, 3.16; P, 3.79.

Examination by thin layer chromatography (solvent B, three developments) showed that the product (R_F 0.46) contained about 2–3% of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-D-glucopyranosyl phosphate ($Ac_3GlcNAc$-*P*) (migrating just ahead of the main spot). In solvent C a single spot (R_F 0.64) was obtained. Compound **1** (16 mg; Fig. 1) was treated with an excess of 1% sodium methoxide in methanol at room temperature for 30 min. After dilution with absolute ethanol (5 ml), a solid precipitated at −15° overnight. It was centrifuged off and washed twice with cold absolute ethanol to yield amorphous compound **2** (11 mg), which decomposed slowly when heated above 180°, $[\alpha]_D^{26}$ −2° [c 0.55, methanol–water (1:1)]; R_F 0.20 (solvent C). Calculated for $C_{16}H_{27}N_2Na_2O_{14}P \cdot 4H_2O$: C, 30.97; H, 5.68; N, 4.51; P, 4.99. Found: C, 30.82; H, 5.18; N, 4.80; P, 5.77.

Examination by thin layer chromatography in solvents C (three developments, R_F 0.60, 0.54) and E (R_F 0.32, 0.25) showed that compound **2** was a 1:1 mixture of α and β anomers and that it contained some 2-acetamido-2-deoxy-D-glucopyranosyl phosphate (GlcNAc-*P*) (less than 5% of total). GlcNAc-*P* and GlcNAc-GlcNAc-*P* were separated by cellulose thin layer chromatography, using for detection either a phosphate-specific spray reagent or chlorine gas–starch–potassium iodide (17); GlcNAc-*P*, R_F 0.17; GlcNAc-GlcNAc-*P*, R_F 0.30. Compound **2** (5 mg) was treated with 0.1 M HCl (1 ml) at 100° for 5 min, when thin layer chromatography (solvent C) showed that more than 80% of the original phosphate had been hydrolyzed. The solution was neutralized with 1% sodium methoxide in methanol and evaporated to dryness. Examination of the residue by thin layer chromatography (solvent B, three developments, and solvent E) showed that the product consisted mainly of di-*N*-acetylchitobiose, together with a small proportion of GlcNAc. It was stirred overnight with acetic anhydride and pyridine (1:2, 1 ml). After the addition of water (0.5 ml), the solution was kept at room temperature for 1 hr and evaporated. After three additions and evaporations of toluene (1 ml), the product was shown, by thin layer chromatography in chloroform–methanol (10:1), to consist of peracetylchitobiose (R_F 0.34) and peracetyl GlcNAc (R_F 0.57) in about an 8:1 ratio.

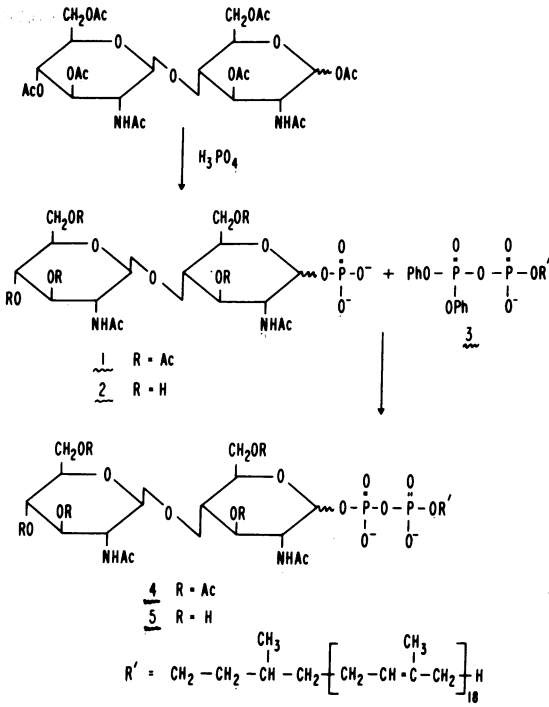

FIG. 1. Synthesis of Dol-*P*-*P*-GlcNAc-GlcNAc.

Synthesis of Dol-P-P-GlcNAc-GlcNAc (*Fig. 1, Structure* **5**). A mixture of $Ac_3GlcNAc$-$Ac_2GlcNAc$-*P* (structure **1**, tributylammonium form, 20 mg) and P^1-diphenyl P^2-dolichyl pyrophosphate (structure **3**, prepared from 14 mg of dolichol) was dried by repeated addition and evaporation of toluene, and dissolved in 1,2-dichloroethane (0.4 ml) containing dry pyridine (4 mg). The mixture was kept at room temperature for 48 hr, diluted with chloroform (10 ml), and extracted three times with water (2 ml). Unreacted $Ac_3GlcNAc$-$Ac_2GlcNAc$-*P* was recovered from the combined aqueous extracts. The chloroform solution was concentrated to 1 ml and compound **4** was purified by preparative thin layer chromatography (solvent A, R_F 0.53), using a phosphate-specific spray to locate the required band. This material was extracted with solvent C by stirring overnight to give Dol-*P*-*P*-$Ac_2GlcNAc$-$Ac_3GlcNAc$ (structure **4**, tributylammonium form, 4 mg). In order to obtain compound **4** in the stable, disodium form, the aqueous chloroform–methanol extract from the silica gel thin layer chromatography plate was treated with 1% sodium methoxide in methanol (2 ml) to give pH 7–8, before evaporation. The infrared spectrum of compound **4** showed peaks arising from the GlcNAc, dolichol, and pyrophosphate residues.

Treatment of compound **4** (sodium form, 4 mg) with an excess of a 1% solution of sodium methoxide in chloroform–methanol (2:1) gave a product shown by thin layer chromatography (solvent B) to contain a single major component, to-

TABLE 1. *Thin layer chromatography of Dol-P-P-GlcNAc and Dol-P-P-GlcNAc-GlcNAc*

Solvent[a]	Dol-*P*-*P*-GlcNAc, R_F[b]	Dol-*P*-*P*-GlcNAc-GlcNAc, R_F
A	0.21	0.09
B	0.64	0.42
D	0.53	0.14[c]
E	0.80	0.77
F	0.25	0.13

[a] For details of solvent systems, see *General Methods*.

[b] R_F values were calculated from measurement of distance from the origin of the chromatogram to the point of maximum intensity of the spot.

[c] With streaking.

gether with traces of Dol-*P*-*P*-GlcNAc and other by-products. Purification was achieved by preparative thin layer chromatography (solvent B) without prior addition of cation exchange resin. The band containing compound **5** was located, and the material was extracted as described for compound **4** to give Dol-*P*-*P*-GlcNAc-GlcNAc (compound **5**, 1 mg), pure according to thin layer chromatography in five solvent systems with spray reagents specific for carbohydrates, lipids, and phosphate esters (see *General Methods*). Compound **5** was well separated by thin layer chromatography from Dol-*P*-*P*-GlcNAc (see Table 1). Calculated ratio of GlcNAc to phosphate = 1:1; found: 1.06:1.

Preparation of Lymphocyte Membranes. Lyophilized material equivalent to 10 ml packed volume of human lymphocyte cell line IM-1 (generously supplied by Dean Mann, NIH) was suspended in 50 ml of 1 mM EDTA and adjusted to pH 8.0 by addition of 1 M Tris base. The suspension was homogenized by 20 passes in a Potter–Elvehjem homogenizor and centrifuged at 2,000 × *g* for 15 min. The pellet was resuspended in 40 ml of 50 mM Tris-maleate (pH 8.0)–1 mM EDTA homogenized, and centrifuged. The combined supernatants were centrifuged at 150,000 × *g* for 1 hr. The pellet was resuspended in 20 ml of Tris-maleate–EDTA, homogenized, and centrifuged again. The pellet was finally suspended in 20 ml of Tris-maleate–EDTA. This gave a preparation containing 5 mg/ml of protein, as determined by the Lowry method with bovine serum albumin as standard.

RESULTS

Synthesis of Dol-P-P-GlcN Ac-GlcN Ac (Fig. 1, Compound **5**). Compound **5** was synthesized as shown in Fig. 1; details of the procedure are given in *Materials and Methods*. Compounds **1**, **2**, and **4** contained a small proportion of the corresponding GlcNAc derivative. This was not present, however, in the chromatographically purified sample of compound **5**.

Compound **4** was not separated from Dol-*P*-*P*-Ac_3GlcNAc by thin layer chromatography, but the two compounds were readily distinguishable by their different stabilities. Whereas the monosaccharide derivative was stable in chloroform–methanol solution as the tributylammonium salt, compound **4** was only stable after conversion into the sodium salt. The decomposition of compound **4** gave rise to Dol-*P*-*P* and an acetylated chitobiose derivative (thin layer chromatography). When a sample of compound **4** had completely decomposed, the products were acetylated and examined by thin layer chromatography, which showed that fully acetylated di- and monosaccharides were present in about a 10:1 proportion. This result confirmed that compound **4** was mainly a derivative of di-*N*-acetylchitobiose.

Compound **5** was easily separated from Dol-*P*-*P*-GlcNAc in a variety of solvent systems (see Table 1). When compound **5** was converted into the pyridinium form, by action of a cation exchange resin, it became very unstable, decomposing to yield Dol-*P*-*P* (thin layer chromatography). In contrast, Dol-*P*-*P*-GlcNAc was stable to this treatment. When a sample of compound **5** was hydrolyzed with hot, dilute alkali in propanol, thin layer chromatography showed that Dol-*P* was a major product, and Dol-*P*-*P* a minor product. Thin layer chromatography also showed a faint spot corresponding to GlcNAc-GlcNAc-*P* in solvents C (three developments) and E.

Effect of Dol-P-P-GlcNAc-GlcNAc on the Formation of a Mannose Compound with the Properties of an Oligosaccharide Phospholipid. In double-labeling experiments, both GDP-[^{14}C]Man (160 mCi/mmol) and UDP-[^{3}H]GlcNAc (6600 mCi/mmol) were incubated with membrane preparations from human lymphocytes under conditions that have been shown to lead to the synthesis of lipid-linked sugars. Under these conditions, a considerable amount of Dol-*P*-Man (region 2) was formed from endogenous acceptor and a much smaller amount of a GlcNAc-containing compound (region 3) was synthesized (Fig. 2A).

On addition of synthetic Dol-*P*, the synthesis of Dol-*P*-Man and of the [^{3}H]GlcNAc-containing compound were greatly stimulated (Fig. 2B). This second compound (region 3) eluted from the DEAE–cellulose column only at salt concentrations much higher than those required for the elution of Dol-*P*-Man, a property which suggests that it is a pyrophosphate diester.

When synthetic Dol-*P*-*P*-GlcNAc was substituted for Dol-*P*, there was stimulation of a new compound which eluted in region 3 and contained both mannose and GlcNAc (Fig. 2C).

In the presence of synthetic Dol-*P*-*P*-GlcNAc-GlcNAc a mannose-containing compound (region 3) was formed (Fig. 2D). In addition, a compound containing GlcNAc was formed, possibly due to traces of Dol-*P*-*P*-GlcNAc in the Dol-*P*-*P*-GlcNAc-GlcNAc preparation. (See further discussion below.) Addition of Dol-*P*-*P* and di-*N*-acetylchitobiose did not stimulate the formation of the mannose-containing compound that was eluted in region 3 (Fig. 2E).

The labeled compounds, that were eluted in region 3, formed in the presence of added Dol-*P*-*P*-GlcNAc (Fig. 2C) or Dol-*P*-*P*-GlcNAc-GlcNAc (Fig. 2D) were hydrolyzed with dilute acid, and the water-soluble products were analyzed by gel filtration on Biogel P-2, with glucose, sucrose, maltotriose, stachyose, and Blue Dextran as internal standards (Fig. 3A and B). The hydrolyzate in both cases contained several [^{14}C]Man compounds, one of which was larger than a tetrasaccharide. It is not clear whether the multiple peaks in either case represent acid degradation of a single released oligosaccharide, or the existence of several different classes of lipid, or a combination of both.

While the [^{3}H]GlcNAc in the first case was associated with the [^{14}C]Man oligosaccharides, in the second case it was not. Instead, the [^{3}H]GlcNAc migrated as a discrete peak approximately in the position expected for a disaccharide. Since the amount of labeled lipid formed is very small (1–10 pmol)

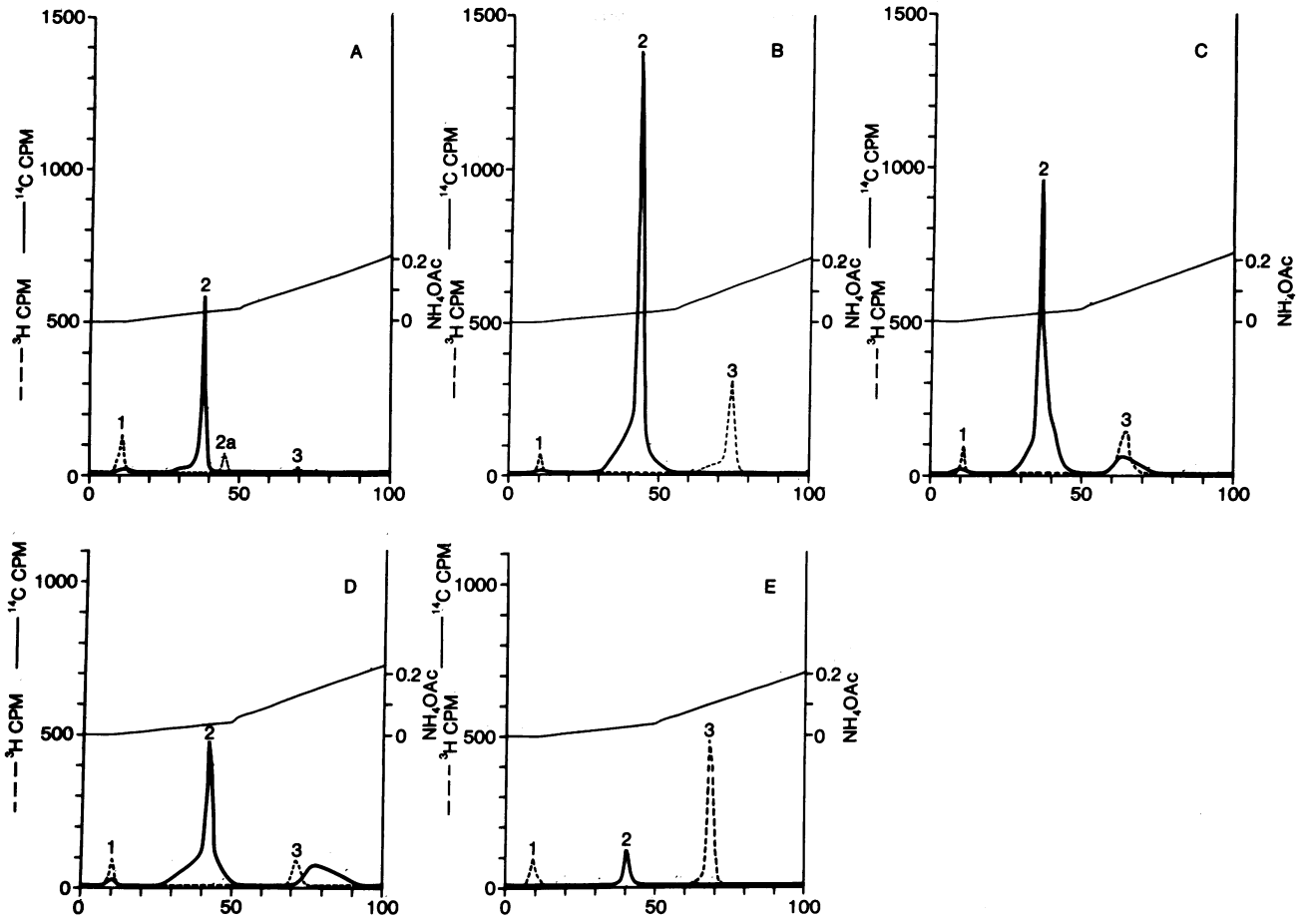

FIG. 2. DEAE–cellulose chromatography of the lipid extracts from (A) endogenous acceptor; (B) with 10 nmol of Dol-*P* added; (C) with 18 nmol of Dol-*P*-*P*-GlcNAc added; (D) with 30 nmol of Dol-*P*-*P*-GlcNAc-GlcNAc added; and (E) with 30 nmol each of Dol-*P*-*P* and di-*N*-acetylchitobiose added.

A standard incubation mixture contained 60 mM Tris-maleate (pH 8.0), 10 mM $MnCl_2$, 1 mM EDTA, 1.5 μM GDP-[^{14}C]Man, and 0.6 μM UDP-[^{3}H]GlcNAc in 1 ml with 3.2 mg of protein from a membrane preparation of human lymphocyte cell line IM-1. Dolichol derivatives were added by drying with 5 mg of Triton X-100 under a stream of nitrogen. The incubation mixture was then added and mixed vigorously on a Vortex mixer. The mixture was incubated at room temperature for 60 min, boiled for 2 min, and extracted 4 times with 2 ml of water-saturated 1-butanol. The combined butanol phases were washed 3 times with water and dried under reduced pressure.

The lipid material was dissolved in 10 ml of chloroform–methanol (1:1) and layered onto a 2.5 × 20-cm column of DEAE–cellulose. The column was then washed with 120 ml of methanol and eluted with two gradients: the first was 300 ml of methanol and 300 ml of 50 mM ammonium acetate in methanol, the second was 500 ml of 50 mM ammonium acetate and 500 ml of 300 mM ammonium acetate. The first gradient was stopped and the second started about fraction 50. Fractions of about 12 ml were collected, and 0.4 ml of each fraction was counted. Relevant fractions were pooled, and 0.5 volume of water and 1.5 volumes of chloroform were added. The lower phase was washed once with 1 volume of methanol–water (1:1) and dried under reduced pressure. The lipid material was then dissolved in chloroform–methanol (1:1) and stored at −20° until use. Region 1 contained nonlipid material. Region 2a contained a lipid whose biosynthesis is extremely sensitive to Triton X-100, and is not stimulated by Dol-*P*.

compared to the amount of synthetic lipid added (10,000–30,000 pmol), this "disaccharide" may represent the transfer of [^{3}H]GlcNAc to trace amounts of Dol-*P*-*P*-GlcNAc either present in the sample of Dol-*P*-*P*-GlcNAc-GlcNAc or formed enzymatically. Any labeled Dol-*P*-*P*-GlcNAc-GlcNAc formed would undergo isotope dilution and, thus, further utilization would be obscured.

Nature of the Mannose-Containing Compound Formed. It was concluded that this [^{14}C]Man-containing derivative eluting in region 3 was an oligosaccharide phospholipid on the basis of (*a*) its stimulation of formation by Dol-*P*-*P*-GlcNAc or Dol-*P*-*P*-GlcNAc-GlcNAc, (*b*) its extraction by 1-butanol from the mixture of incubation products, (*c*) its presence in the chloroform-rich phase in the Folch extraction, (*d*) its adsorption onto DEAE–cellulose and elution at a high salt concentration, and (*e*) its hydrolysis by dilute acid to give a compound with gel-filtration properties of an oligosaccharide.

Role of GDP-Man in the Formation of the Oligosaccharide Phospholipid. To examine this question, Dol-*P*-[^{14}C]Man was used as a source of mannose instead of GDP-[^{14}C]Man. No mannose-containing oligosaccharide derivative was formed in the presence or absence of Dol-*P*-*P*-GlcNAc-GlcNAc when this source of mannose residues was used (Fig. 4A and B).

DISCUSSION

The chemical synthesis of a disaccharide polyisoprenyl pyrophosphate, Dol-*P*-*P*-GlcNAc-GlcNAc, has been described. Pyrophosphate diesters derived from GlcNAc and di-*N*-acetyl-

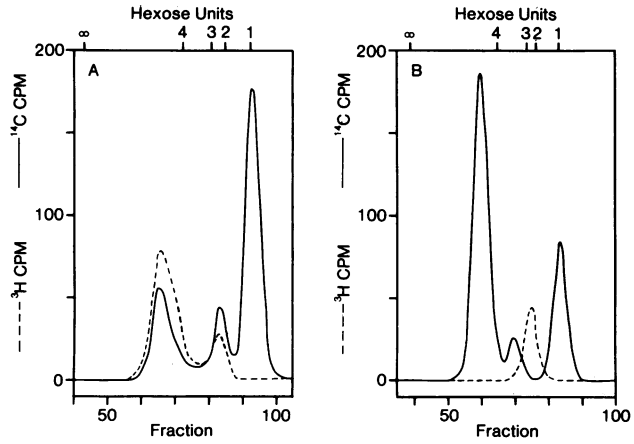

FIG. 3. Gel filtration of lipid hydrolysates. (A) From region 3, Fig. 2C; (B) from region 3, Fig. 2D. A sample of lipid was dried under a stream of nitrogen, suspended in 200 μl of 0.1 M HCl, and heated at 105° for 15 min. After extraction with butanol, the pH was adjusted to 5 with sodium acetate, and 1 mg of Blue Dextran, 1.5 mg of stachyose, 0.4 mg of maltotriose, 2.5 mg of sucrose, and 0.2 mg of glucose were added in a final volume of 350 μl. The entire sample was applied to a 0.9 × 95-cm column of Biogel P-2 in 10 mM ammonium bicarbonate. Fractions of 0.60 ml were collected, and 0.5 ml was counted. Blue Dextran, stachyose, and sucrose were determined by the anthrone assay, while maltotriose and glucose were determined by the reducing sugar assay.

chitobiose were well separated by thin layer chromatography. The effect of an additional sugar residue on the chromatographic mobility was a marked one, despite the presence of a pyrophosphate group and a very long (C_{95}) aliphatic chain.

Experiments presented herein have shown that synthetic Dol-*P*-*P*-GlcNAc-GlcNAc (compound **5**) is required by membrane preparations from human lymphocytes for the formation of a mannose-containing compound with the properties of an oligosaccharide phospholipid, the oligosaccharide moiety of which contains more than four sugar residues. A similar oligosaccharide phospholipid containing, in addition, labeled GlcNAc was formed on addition of Dol-*P*-*P*-GlcNAc. Presumably the GlcNAc is transferred first to give Dol-*P*-*P*-GlcNAc-[^{3}H]GlcNAc to which mannose is then added. The fact that GDP-Man is a mannosyl donor to Dol-*P*-*P*-GlcNAc-GlcNAc, while Dol-*P*-Man does not appear to be, may suggest that mannosyl residues at different positions in the glycoprotein have different anomeric configurations and that different mannosyl donors function in their synthesis.

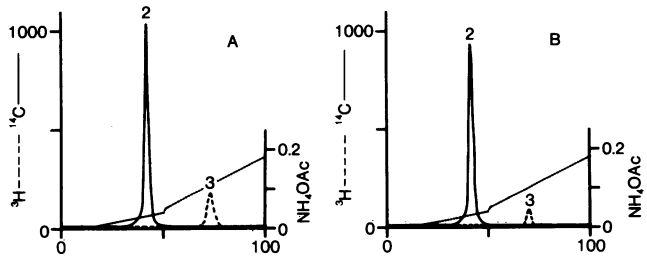

FIG. 4. DEAE-cellulose chromatography. A, endogenous acceptor with Triton X-100 present; B, with 30 nmol of Dol-*P*-*P*-GlcNAc-GlcNAc and Triton X-100 present. The same as Fig. 2, except that Dol-*P*-[^{14}C]Man (150,000 cpm) instead of GDP-[^{14}C]Man was added.

Other workers have observed the formation of similar lipid-bound oligosaccharide phosphates using enzyme preparations from rat liver (7), mouse myeloma (8), and hen oviduct (9). Although a role for Dol-*P*-*P*-GlcNAc-GlcNAc as an acceptor of mannose residues in the formation of these compounds has been suggested (8), a direct demonstration of its requirement has not been reported.

In some instances oligosaccharide phospholipids have been shown to function as lipid intermediates in glycoprotein biosynthesis (3, 8). It is possible that the compound described herein has a similar activity.

Note Added in Proof. Recently, mannosyl transfer to an endogenous di-*N*-acetylchitobiose-containing lipid to form oligosaccharide lipid with hen oviduct or rat liver microsomes has also been demonstrated (18). These authors also observed the failure of Dol-*P*-Man to substitute for GDP-Man in the initial mannosyl transfer.

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