BIOSYNTHESIS OF A P^1-2-ACETAMIDO-2-DEOXY-D-GLUCOSYL P^2-POLYISOPRENYL PYROPHOSPHATE BY CALF PANCREAS MICROSOMES*

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SUMMARY: Incubation of UDP-[^{14}C]-N-acetylglucosamine with calf pancreas microsomes in the presence of Mn^{++} and potassium thiocyanate gave a labeled glycolipid, tentatively identified as P^1-2-acetamido-2-deoxy-D-glucosyl P^2-dolichyl pyrophosphate on the basis of cochromatography with synthetic P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate, similar chemical and enzymic hydrolyses of the biosynthetic and synthetic compounds, and stimulation of the biosynthesis by addition to the incubation mixture of dolichyl phosphate or a crude lipid fraction extracted from microsomes.

Biosynthesis of GlcNAc derivatives of polyisoprenyl phosphates by incubation of UDP-[^{14}C]-GlcNAc with rat and rabbit liver microsomes, and an enzyme system from Staphylococcus lactis have been reported (1-9), but no direct evidence concerning the identity of the polyisoprenyl residue has been presented. The possible involvement of a dolichyl phosphate derivative was suggested by the stimulation of the biosynthesis of the glycolipid by dolichyl phosphate and a crude lipid fraction obtained from microsomes (3,7). The present report identifies the GlcNAc-containing lipid as P^1-2-acetamido-2-deoxy-D-glucosyl P^2-polyisoprenyl pyrophosphate, the polyisoprenyl residue being very similar to or identical with a dolichyl residue.

MATERIALS. UDP-[^{14}C]-GlcNAc (275 Ci/M) was purchased from Amersham/Searle; Silica Gel G-60 (75-235 mesh), Silica Gel F254, precoated Silica Gel G thin-layer plates and plates for preparative tlc were purchased from E. Merck

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(Darmstadt); DEAE-cellulose (DE52-preswollen, microgranular) from W. R. Balston, Inc. (Lexington, Mass.); calf intestine alkaline phosphatase from Boehringer, Mannheim Corp. (New York); phosphodiesterase I from Pierce Chemical Co. (Rockfort, Ill.); and soybean trypsin inhibitor from Worthington Biochemical Corp. (Freehold, N.J.). Dolichyl phosphate, other polyisoprenyl phosphates, and their sugar derivatives were synthesized in this laboratory (10-12).

METHODS. Preparation of the Microsomal Fraction. Unless indicated otherwise, all procedures were performed at 4°. Calf pancreas was cleaned, washed with buffer (10mM Tris-maleate, pH 7.3, containing 2mM $MgCl_2$ and 0.25M sucrose), and homogenized in buffer (4 vol.) containing 0.02% soybean trypsin inhibitor. The homogenate was centrifuged at 10,000g for 10 min, the supernatant fluid ultracentrifuged at 100,000g for 1 hr, and the pellet (microsomal fraction) stored at -70°. For routine assays, the microsomal preparation was suspended in 0.05M Tris-maleate, pH 7.3 at a protein concentration of 20 mg/ml.

Standard Assay Procedure. The incubation mixture (final vol. 250 µl) contained 50mM Tris-maleate (pH 7.3), 0.25µM (28,000 cmp) UDP-[^{14}C]-GlcNAc, 10mM $MnCl_2$, 0.2M KCNS, and microsomal protein (3 mg). After a 5-min incubation at 32°, 6% CCl_3COOH containing 0.25% phosphotungstic acid (4 vol.) was added and mixed vigorously. After centrifugation, the precipitate was washed again with CCl_3COOH and extracted with $CHCl_3$-CH_3OH (2 ml, 2:1, v/v) at room temperature for 10 min, and centrifuged off. The precipitate was dried and dissolved by heating in 0.2M NaOH at 100° for 2 min. The radioactivity of the $CHCl_3$-CH_3OH extract and the alkaline solution was determined (Packard Liquid Scintillation Spectrometer).

Isolation of the Lipid Fraction from Microsomes. A homogenate of microsomes (100 mg) in 0.1M Tris-maleate containing 5mM $MgCl_2$ and 0.2M KCNS was incubated at 30° for 1 hr, then at 68° for 15 min, and centrifuged at 30,000g for 30 min. The supernatant fluid was lyophilized, and the residue extracted with $CHCl_3$-CH_3OH (2:1) and dried in vacuo. The extract was chromatographed

on a Silica Gel column and eluted with $CHCl_3$-CH_3OH (2:1).

Chemical and Enzymic Hydrolyses of Glycolipid. A sample containing biosynthetic (2000 cpm) and synthetic glycolipid (75 µg), dried under N_2, was hydrolyzed in 0.1M HCl at 100° for 10 min or in $CHCl_3$-CH_3OH-H_2O (1:1:0.3) containing 0.2M NaOH at 68° for 1 hr. In either case, $CHCl_3$ and CH_3OH were added to the hydrolyzate to attain a concentration of 2:1, and the solutions extracted with water (0.2 vol.).

Another sample of biosynthetic (2000 cpm) and synthetic glycolipid (150 µg) was dried under N_2 and treated with phosphodiesterase I (2 mg) in 0.1M Tris-acetate (pH 8.5) containing 30mM $Mg(OAc)_2$ and 0.1M KCNS. The product of alkaline hydrolysis of the mixture of glycolipids was treated with alkaline phosphatase (5 mg) in 40mM glycine buffer (pH 9.5) containing 10mM $MgCl_2$.

In each case, the compounds extracted in the aqueous phase were analyzed by tlc after the addition of $CHCl_3$ and CH_3OH to a final concentration of 2:1.

Chemical Synthesis of P^1-2-Acetamido-2-deoxy-α-D-glucopyranosyl P^2-Dolichyl Pyrophosphate. Dolichyl phosphate (10) (tributylammonium form) was converted, by an adaptation of the method (12) previously used for ficaprenyl phosphate, into the P^1-diphenyl P^2-dolichyl pyrophosphate, which was treated with 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl phosphate in the tributylammonium form, to give a fully acetylated pyrophosphate diester. This was purified by preparative tlc in $CHCl_3$-CH_3OH-H_2O (60:25:4) and de-O-acetylated by treatment with 0.5% sodium methoxide in CH_3OH-$CHCl_3$ (1:1), followed by removal of the excess of sodium ions with Dowex 50 (pyridinium form). The infrared spectrum corresponds to the expected structure.

RESULTS AND DISCUSSION. Biosynthesis of Glycolipid. The rate of incorporation of radioactivity into the glycolipid upon incubation of UDP-[^{14}C]-GlcNAc with pancreas microsomes was rapid, ca. 5 min, then decreased, while the radioactivity of the CCl_3COOH-insoluble material increased (Fig. 1). The

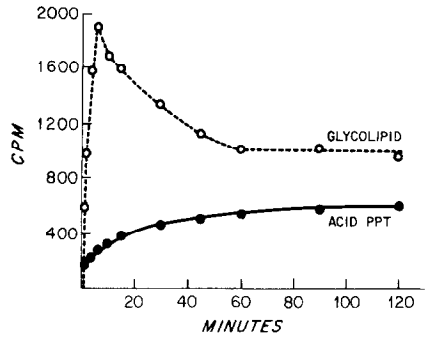

Fig. 1. Incorporation of [^{14}C]-GlcNAc into the glycolipid (o----o) and the CCl_3COOH-insoluble material (●——●). The glycolipid was dissolved in $CHCl_3$-CH_3OH (2:1) and the CCl_3COOH-insoluble material in 0.2N NaOH. The conditions of the experiment were the same as described in "METHODS".

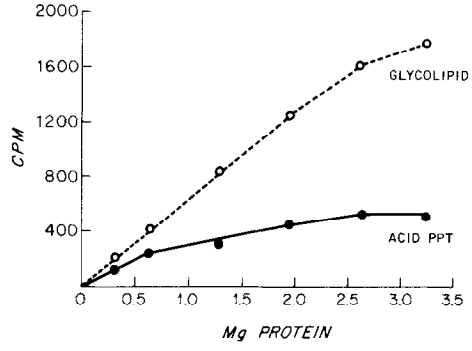

Fig. 2. Effect of microsomal protein concentration on the incorporation of radioactivity into the glycolipid (o----o) and the CCl_3COOH-insoluble material (●——●). The procedure is described under "METHODS". The protein content was determined by the method of Lowry et al. (13).

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rate was dependent upon microsomal protein concentration (Fig. 2); it was maximal at pH 7.1 in the presence of 10mM of Mn^{++} or Mg^{++}. The activity was inhibited by EDTA and by Triton X-100, but stimulated by KCNS (14), up to 3-fold at a 0.2M concentration.

Addition to the incubation mixture of dolichyl phosphate and of phosphate derivatives of ficaprenol and solanesol at higher concentrations stimulated the biosynthesis of both the glycolipid and CCl_3COOH-insoluble product,

TABLE I. Stimulation of incorporation of radioactivity into the glycolipid by polyisoprenyl phosphates[a]

Compounds	Concentration (μM)	Stimulation of incorporation
None	0	1
Citronellyl phosphate	420	$\leqslant 1$
Farnesyl phosphate	420	$\leqslant 1$
Solanesyl phosphate	370	1.6
Ficaprenyl phosphate	270	2.6
Dolichyl phosphate	55	5.3

[a]The conditions are described under "Standard Assay Procedure". The polyisoprenyl phosphates were added in the presence of 0.1% Triton X-100.

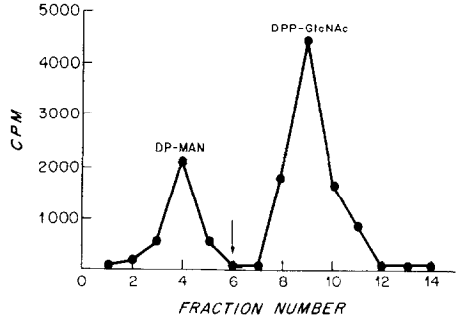

Fig. 3. Elution of the ^{14}C-labeled synthetic dolichyl α-D-mannopyranosyl phosphate (DP-MAN) and a mixture of synthetic P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate (DPP-GlcNAc) and biosynthetic glycolipid from a DEAE-cellulose acetate column performed according to Leloir et al. (7). The arrow indicates the start of elution with ammonium formate.

TABLE II. Thin-layer chromatography of glycolipids

Compounds	R_{GlcNAc}[a]			
	A	B	C	D
DPP-GlcNAc[b]	1.83	3.90	2.00	1.56
Glycolipid + DPP-GlcNAc	1.85	3.95	2.05	1.55
FPP-GlcNAc[c]	1.32	3.05	1.60	1.28
Dolichyl phosphate	3.30	5.00	2.35	2.75

[a]The chromatography was performed on thin layers of Silica Gel G (Merck) in the following solvent systems: (A) $CHCl_3$-CH_3OH-H_2O (60:25:4); (B) 2,6-dimethyl-4-heptanone-HOAc-H_2O (20:15:2); (C) $CHCl_3$-CH_3OH-conc. NH_4OH-H_2O (65:35:4:4); (D) $CHCl_3$-CH_3OH-H_2O (60:35:6). Compounds were detected with the anisaldehyde spray (15), the potassium permanganate spray reagent (16), and phospholipids by the method of Dittmer and Lester (17).

[b]DPP-GlcNAc, synthetic P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate.

[c]FPP-GlcNAc, synthetic P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-ficaprenyl pyrophosphate (12).

whereas derivatives of farnesol and citronellol were inactive (Table I). The incorporation of radioactivity into the glycolipid was stimulated (up to 5 times) by addition of the crude lipid fraction to the incubation mixture.

Properties of the Glycolipid. The radioactive glycolipid cochromatographed with synthetic P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate on a column of Silica Gel eluted with $CHCl_3$-CH_3OH (2:1), and of DEAE-cellulose eluted with 25mM NH_4-formate (7) (Fig. 3), and on tlc plates (Table II). The main products of acid and alkaline hydrolyses of both biosynthetic and synthetic glycolipids were GlcNAc and GlcNAc 1-phosphate, respectively, the latter compound giving GlcNAc when hydrolyzed by phosphatase (Table III). Treatment of the mixture of biosynthetic and synthetic compounds with phosphodiesterase gave one radioactive spot migrating on tlc with GlcNAc 1-phosphate (Table III).

TABLE III. Thin-layer chromatography of products of hydrolysis of the glycolipid in the presence of acid, base, and phosphodiesterase

Compounds	R_{GlcNAc}[a]				
	A	B	E	F	G
D-Glucosamine	0.0	0.25	0.67	0.50	0.78
GlcNAc phosphate	0.0	0.21	0.30	0.34	0.30
Product of acid hydrolysis	1.00	1.00	1.00	1.00	—
Product of alkaline hydrolysis	0.0	0.20	0.30	0.33	—
Product of alkaline hydrolysis after phosphatase treatment	1.00	0.96	1.02	1.00	—
Product of phosphodiesterase treatment	0.0	0.21	0.31	0.34	0.30

[a]The chromatography was performed on thin layers of Silica Gel G (Merck) in the following solvent systems: (A) $CHCl_3$-CH_3OH-H_2O (60:25:4); (B) 2,6-dimethyl-4-heptanone-HOAc-H_2O (20:15:2); (E) $CHCl_3$-CH_3OH-H_2O (1:1:0.3); (F) 2-propanol-conc. NH_4OH-H_2O (6:3:1); and (G) 1-propanol-conc. NH_4OH (1:1). The compounds were detected as described in Table II.

The results indicate that microsomal preparations of calf pancreas catalyze the incorporation of [^{14}C]-GlcNAc from UDP-[^{14}C]-GlcNAc into a glycolipid tentatively identified as P^1-2-acetamido-2-deoxy-D-glucosyl P^2-dolichyl pyrophosphate. The pyrophosphate bond is ascertained by the formation of GlcNAc 1-phosphate by alkaline hydrolysis and by phosphodiesterase action from both the natural glycolipid and the synthetic P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-dolichyl pyrophosphate, and by the chromatographic behavior of these compounds on DEAE-cellulose (7). The dolichyl nature of the isoprenyl moiety is suggested by the cochromatography of both biosynthetic and synthetic glycolipids on columns and thin-layer plates in several solvent systems, and by the stimulation of the glycolipid formation by addition of dolichyl phosphate to the incubation mixture. The presently reported biosynthesis of the glycolipid from dolichyl phosphate and UDP-GlcNAc with the

release of UMP is similar to the formation of GlcNAc-containing polyisoprenyl pyrophosphates in Staphylococcus lactis (8,9) and mammalian systems (7,18,19).

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