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BIOSYNTHESIS OF A D-GLUCOSYL POLYISOPRENYL DIPHOSPHATE IN PARTICULATE PREPARATIONS OF *Micrococcus lysodeikticus*[*,†]

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ABSTRACT

Particulate fractions of *Micrococcus lysodeikticus* incubated with UDP-D-[^{14}C]glucose incorporated radioactivity into a chloroform–methanol-soluble, low-mol. wt. compound, and into a polymer. The low-mol. wt. compound consisted of a glucolipid that was extremely labile to mild acid hydrolysis with the formation of D-[^{14}C]glucose, and to mild alkali, yielding ^{14}C-labeled α-D-glucopyranose 1,2-phosphate and D-glucose 2-phosphate. The labeled glucolipid was eluted from a DEAE-cellulose column at a salt concentration higher than that required by synthetic ficaprenyl (D-glucopyranosyl phosphate), and it migrated more slowly than the latter compound in t.l.c. Formation of the glucolipid was stimulated by exogenous ficaprenyl phosphate, but not by C_{55}-dolichyl phosphate. These results suggest that the [^{14}C]glucolipid has the characteristic properties of a polyisoprenyl glucosyl diphosphate.

INTRODUCTION

The cell wall of *Micrococcus lysodeikticus* contains two major polymers, a peptidoglycan and an acidic polysaccharide (teichuronic acid). The latter consists of the disaccharide repeating unit (1→4)-*O*-(2-acetamido-2-deoxy-α-D-mannopyranosyluronic acid)-(1→6)-*O*-β-D-glucopyranosyl[3–5]. Some experimental evidence has suggested that the teichuronic acid chains are covalently attached to the

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peptidoglycan chain by a phosphate diester linkage between C-1 of the terminal, potentially reducing D-glucose residue of the polysaccharide chain[5], and C-6 of a muramic acid residue. On the other hand, other work has suggested that the teichuronic acid chains are linked to muramic acid 6-phosphate residues of the peptidoglycan through a 2-acetamido-2-deoxy-D-glucosyl residue[6]. Thus, the exact nature of the linkage region remains unclear, and it has not been determined whether or not these differing results arise from heterogeneity in the mode of attachment of the polysaccharide to the peptidoglycan backbone.

The biosynthesis of teichuronic acid from labeled uridine 5′-(α-D-glucopyranosyl diphosphate) (UDPGlc) and labeled uridine 5-(2-acetamido-2-deoxy-α-D-mannopyranosyluronic acid diphosphate) (UDPManANAc) in particulate fractions of *M. lysodeikticus* has been demonstrated; small amounts of uridine 5′-(2-acetamido-2-deoxy-α-D-glucopyranosyl diphosphate) (UDPGlcNAc) were required to observe the synthesis[7]. It was subsequently shown that the first stage of biosynthesis is the formation, by sequential addition of the monosaccharide residues[8], of a lipid intermediate containing one 2-acetamido-2-deoxy-D-glucosyl residue at the (potentially) reducing-end, and two residues of 2-acetamido-2-deoxy-D-mannosyluronic acid. In the second stage, this lipid intermediate acts as an acceptor of alternating D-glucose and 2-acetamido-2-deoxy-D-mannuronic acid units[9]. It has also been reported that the biosynthesis of teichuronic acid is inhibited by bacitracin and tunicamycin, both of which inhibit the formation of lipid intermediates[10]. In these studies, however, there was no evidence for the formation of a lipid intermediate containing D-glucose at the (potentially) reducing end. In the present work, we demonstrate that particulate preparations of *M. lysodeikticus* incorporate labeled D-glucose from UDPGlc into a glucolipid having the properties of a polyisoprenyl D-glucosyl diphosphate. For the purpose of identifying this glucolipid, the chemical syntheses of ficaprenyl (α- and β-D-glucopyranosyl phosphate) were performed.

EXPERIMENTAL

Materials. — The following compounds were obtained from the companies indicated: UDP-D-[U^{14}-C]glucose (specific activity, 280 mCi/mmol) from New England Nuclear, Boston, MA 02118; AG 50W-X8 and AG IX-8 ion-exchange resins from Bio-Rad Laboratories, Rockville Ctr., NY 11570; DEAE-cellulose (Whatman DE-32) from Whatman Inc., Clifton, NJ 07014; bovine serum albumin from Miles Laboratories, Elkhart, IN 46514; UDP-D-glucose and α-D-glucopyranosyl phosphate (D-glucose 1-phosphate) from Sigma Chemical Co., St. Louis, MO 63118; other sugars from Pfanstiehl Laboratories, Waukegan, IL 60086; Triton X-100 (B grade) from Calbiochem-Behring Corp., Somerville, NJ 08876; Bactopeptone and yeast extract from Difco Laboratories, Detroit, MI 48201; and triisopropylbenzenesulfonyl chloride from Aldrich Chemical Co., Milwaukee, WI 53210. Ficaprenol was isolated from *Ficus elastica* as described by Warren and

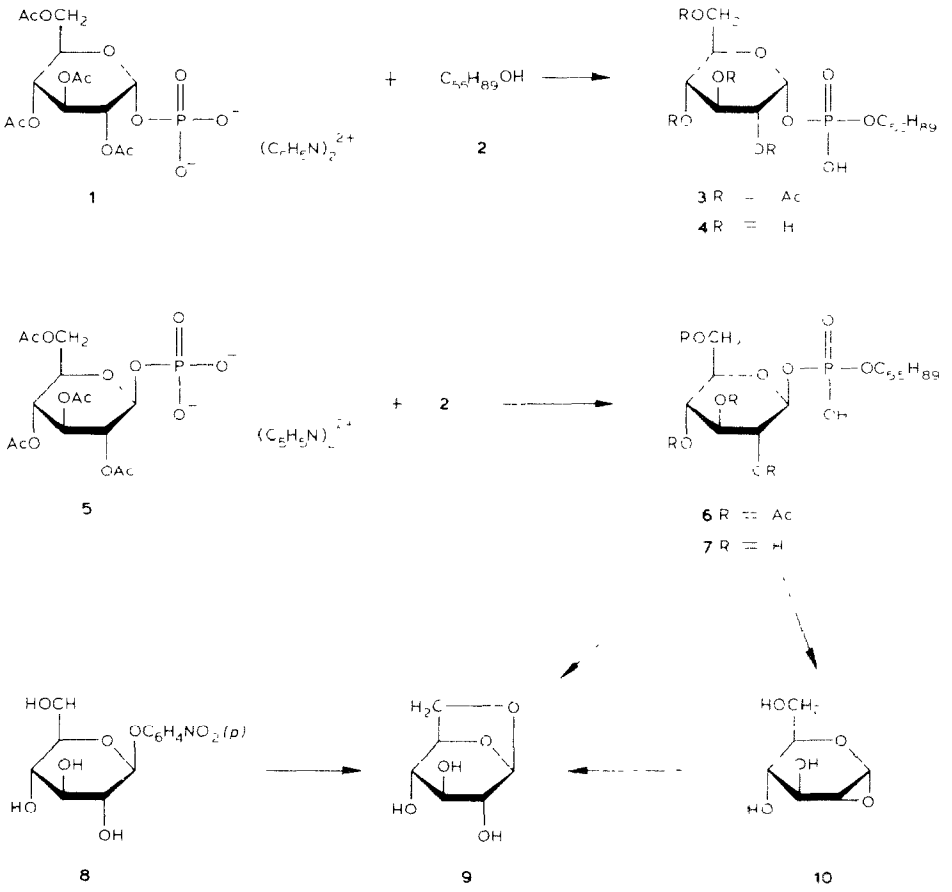
Jeanloz[11]; ficaprenyl phosphate was synthesized as described by Warren *et al.*[12]; and UDP-ManANAc as described by Yamazaki *et al.*[13]. C_{55}-Dolichyl phosphate was a gift from the Polish Chemical Reagent Co., Gliwice, Poland.

Analytical methods. — Protein was estimated by the method of Lowry *et al.*[14] with bovine serum albumin as standard.

Thin-layer chromatography was performed on precoated plates of Silica gel G (0.25 mm thick without fluorescent indicator) obtained from E. Merck AG (Darmstadt, W. Germany) in the following solvents: (*A*) 60:25:4 chloroform–methanol–water, (*B*) 60:35:6 chloroform–methanol–water, (*C*) 20:12:3 chloroform–methanol–water, and (*D*) 20:15:2 2,6-dimethyl-4-heptanone–acetic acid–water. Preparative t.l.c. was performed on plates of Silica gel 60 F254, (0.5 mm thick, Merck), and descending paper chromatography on Whatman No. 1 paper in the following solvents: (*E*) 6:4:3 butanol–pyridine–water, (*F*) 8:2:1 ethyl acetate–pyridine–water, and (*G*) 5:2 95% ethanol–M ammonium acetate, pH 7.5. All solvent systems were v/v. Compounds were detected on thin-layer chromatograms with 5% phosphomolybdic acid in ethanol or with 1:1:18 anisaldehyde–sulfuric acid–ethanol[15]. Sugars were detected on paper chromatograms with the alkaline silver nitrate reagent[16].

Radioactivity was determined with a Packard liquid scintillation spectrometer, Model 3255, for solutions in minivials containing Hydrofluor (3 mL) (National Diagnostics, Somerville, NJ 08876). Organic solvents were evaporated from the samples before counting. Radioactive substances were located on thin-layer chromatograms with Kodak X-Omat XR5 film. Paper chromatograms were scanned with a Packard Radiochromatogram Scanner, Model 7220/21.

Ficaprenyl (α-D-glucopyranosyl phosphate) (**4**). — A mixture of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl pyridinium phosphate[17] (**1**, 20 mg) and ficaprenol (**2**, 35 mg) was dried by repeated addition and evaporation of dry toluene, and then treated with a solution of triisopropylbenzenesulfonyl chloride (12 mg) in dry pyridine (1.0 mL). The reaction tube was sealed, and the contents mixed thoroughly and kept for 48 h at room temperature. After being treated with methanol (0.5 mL), the mixture was kept overnight at room temperature, and the solvents were evaporated (N_2 gas). Toluene (1 mL) was added and evaporated twice, and the residue was dissolved in 5:1 chloroform–methanol (0.5 mL). The solution was applied to two preparative thin-layer plates that were eluted with solvent *A*. The band corresponding to ficaprenyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate (**3**, R_F 0.8) was located by spraying a narrow band with (a) a solution of 1% aqueous, potassium permanganate in 2% aqueous sodium hydrogencarbonate, and (b) a phosphate-specific reagent[18]. The product was extracted from the gel by stirring overnight with 10:10:3 chloroform–methanol–water, and filtration and evaporation gave a syrup (23 mg) showing a double spot when analyzed by t.l.c. with 5:1 chloroform–methanol, and having R_F 0.44. *O*-Deacetylation was performed with an excess of 1% sodium methoxide in dry methanol, plus an equal volume of dry dichloromethane to give a clear solution.



After 30 min, the excess of base was removed by the addition of Ag 50W-X8 cation-exchange resin (pyridinium$^+$), and evaporation gave ficaprenyl α-D-glucopyranosyl phosphate, contaminated by traces of other compounds. Final purification was achieved by preparative t.l.c. in solvent *A*, with detection as described for the peracetyl compound, to give **4** (8 mg), R_F 0.25 (*A*), 0.62 (*B*), and 0.42 (*D*); ν_{max}^{film} 3300 (broad), 2730, 1665, 1450, 1375, 1225, 1150 (v broad), and 880 cm^{-1}.

Anal. Calc. for $C_{61}H_{101}O_9P$: C, 72.54; H, 10.08. Calc. for $C_{61}H_{101}O_9P \cdot CHCl_3 \cdot H_2O$: C, 64.94; H, 9.14. Found C, 64.74; H, 9.17

Ficaprenyl (β-D-glucopyranosyl phosphate) (**7**). — The coupling of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl phosphate[19] (**5**) with ficaprenol, isolation of product **6**, and *O*-deacetylation, were performed as described for **4**. Mobility in t.l.c. of **7** was slightly lower than that of the α-D anomer **4** in most solvent systems: R_F 0.23 (*A*), 0.61 (*B*), and 0.41 (*D*). The best t.l.c. separation of α-D and β-D anomers was by two elutions with 65:35:4:4 chloroform–methanol–conc.ammonium hydroxide–water, R_F 0.45 (**4**), 0.42 (**7**); ν_{max}^{film} identical to that of **4**.

Anal. Calc. for $C_{61}H_{101}O_9P \cdot CHCl_3$: C, 65.97; H, 9.18. Found: C, 66.12; H, 9.17.

Treatment with dilute alkali of **4** *and* **7**. — A mixture of **4** or **7** (0.1 mg), propanol (100 μL), and M sodium hydroxide (10 μL) was kept at 65°. After time intervals of 5 min, 10 min, 2 h, and 4 h, aliquots were withdrawn and analyzed by t.l.c. in solvent *A*.

Preparation of particulate fraction[20]. — *Micrococcus lysodeikticus* ATCC 4698 was grown at 35° in 600-mL batches of medium containing Bactopeptone (0.5%), yeast extract (0.2%), sodium chloride (0.5%), and D-glucose (0.5%), with a doubling time of ~3 h. The incubation was performed in 1500-mL baffled flasks with vigorous shaking. The cells were harvested by centrifugation at about two-thirds of the log phase. All subsequent operations were performed at 0–4° as described by Page and Anderson[7]. The cells obtained from ~5 L of culture medium (~8 g wet-weight) were first washed with 0.5% sodium chloride (80 mL), and then with 0.05M Tris-HCl buffer (80 mL), pH 8.2, containing 0.1M magnesium chloride and 2mM 2-mercaptoethanol (TMM buffer). The cells could be stored in the frozen state without any loss of activity at this stage. The cells were ground with three times their weight of alumina (24 g), and the resulting paste was suspended in TMM buffer (80 mL). The suspension was centrifuged twice at 2000*g* for 10 min, and the supernatant solutions were pooled and centrifuged at 12 000*g* for 10 min. The 12 000*g* supernatant was centrifuged at 85 000*g* for 90 min to give a pellet that was suspended in TMM buffer, and the mixture was centrifuged at 100 000*g* for 60 min. The washed pellet could be stored at −80° for at least one month without loss of activity.

Incubation with UDP-[^{14}*C]glucose.* — For standard conditions, the particulate fraction (0.5–1.0 mg of protein) was incubated in duplicate assays, for 30 min at 25°, in a final volume of 50 μL containing 50mM Tris-maleate buffer, pH 6.5 or 7.8 (as indicated), 30mM magnesium chloride, and 0.01mM UDP-D[^{14}C]glucose, which had been diluted with unlabeled UDP-D-glucose to obtain a specific activity of 100 Ci/mol. At the end of the incubation period, 2:1 chloroform–methanol (1 mL) and water (150 μL) were added. The suspension was thoroughly mixed, and the organic phase was collected ("chloroform–methanol extract"). The residual pellet was washed twice with 1-butanol (1 mL), and twice with 1 mL of water. It was successively extracted with 1-mL and 0.5-mL portions of 10:10:3 chloroform–methanol–water and these two extracts were combined ("chloroform–methanol–water extract"). The lipid-extracted residue was dissolved in 0.5M sodium hydroxide (1 mL), and the solution kept for 30 min at 90°. The radioactivity incorporated into the lipid extracts and into the residue was determined.

When exogenous polyprenyl phosphates were included in the incubation medium, the appropriate amount of polyprenyl phosphate in 2:1 chloroform–methanol was first dried under nitrogen, and then redissolved in 0.5% Triton X-100 (10 μL). The other components of the incubation mixture were then added as just described.

Strong-acid hydrolysis. — The chloroform–methanol extract was dried under nitrogen and heated for 4 h at 100° with 3M hydrochloric acid (200μL). After dilu-

tion with water (5 mL), the hydrolyzate was lyophilized and subjected to paper chromatography with standard sugars in solvents *E* and *F*. The delipidated residue was dried in air and heated for 6 h at 100° with 0.5M sulfuric acid (2 mL). The hydrolyzate was passed through coupled columns of AG 50W-X8 (H^+, 200–400 mesh), and AG 1-X8 (HCO_2^-, 200–400 mesh) ion-exchange resins, which were eluted with water. The eluates were chromatographed with standard sugars in solvent *F*, and the paper scanned for radioactivity.

Mild-acid and mild-alkali treatment. — The chloroform–methanol extract obtained from incubation with UDP-D-[^{14}C]glucose under standard conditions was treated with 0.1M hydrochloric acid or with 0.1M sodium hydroxide in 2:1 chloroform–methanol at room temperature. At various time-intervals, a sample of the chloroform–methanol extract was taken and made neutral with 0.5M sodium hydroxide (0.2 vol.) or 0.5M hydrochloric acid, respectively. After mixing and centrifugation, the radioactivity in each of the two phases was determined.

In some cases, the chloroform–methanol extract was dried under nitrogen and treated with 2M ammonium hydroxide in methanol for 2 h at 37°. The mixture was then chromatographed on paper in solvent *G*, with standards of D-glucose and α-D-glucopyranosyl phosphate. For comparison, a sample of UDP-D-[^{14}C]glucose was treated with 2M ammonium hydroxide for 2 h at 37° and chromatographed in the same solvent; under these conditions, labeled D-glucose 2-phosphate and α-D-glucopyranose 1,2-phosphate are the expected products[21].

RESULTS

*Synthesis and properties of ficaprenyl α- (**4**) and β-D-glucopyranosyl phosphate (**7**).* — Ficaprenol (**2**) was isolated, as described previously[11,12], from the leaves of *Ficus elastica* as a mixture of isoprenologs with the C_{55} compound preponderant. Ficaprenol (**2**) was coupled with 2,3,4,6-tetra-*O*-acetyl α- (**1**) or β-D-glucopyranosyl phosphate[17,19] (**5**) in the presence of anhydrous pyridine and triisopropylbenzenesulfonyl chloride, with strict exclusion of moisture. Analysis of the mixture by t.l.c. revealed the formation of two major compounds having closely similar R_f values, but only one of them was a derivative of per-*O*-acetyl-D-glucopyranosyl phosphate, as shown by treatment of a small portion of the mixture with a solution of sodium methoxide in dry methanol. This caused *O*-deacetylation of the required compound to give a product having a much lower R_f value in t.l.c., whereas the by-product was unaffected. The formation of a major side-product in this type of coupling reaction has been reported previously[22]. In order to separate and purify the required compound, the mixture was processed in three stages: (a) the required product and by-product were isolated together by preparative-layer chromatography, (b) the two compounds were treated with a solution of sodium methoxide in anhydrous methanol, and (c) the required, *O*-deacetylated compound was purified by preparative t.l.c.

As prepared by this method, ficaprenyl α-D- (**4**) and -β-D-glucopyranosyl

phosphate (**7**) were homogeneous according to t.l.c. in a variety of solvent systems, and their structures were confirmed by their i.r. spectra and elemental analyses.

The α-D (**4**) and β-D anomers (**7**) were readily distinguished from each other by treament with hot, dilute alkali. After 5 min, **4** was unchanged, but **7** was hydrolyzed at 80% to yield ficaprenyl phosphate and a compound having an R_F value higher than that of 1,6-anhydro-β-D-glucopyranose (**9**). This compound also resulted from similar treament of *p*-nitrophenyl β-D-glucopyranoside (**8**), whereas prolonged treament (2–5 h) of **7** or **8** yielded **9**. It may therefore be reasonably concluded that the intermediate compound was 1,2-anhydro-α-D-glucose (**10**), formed by nucleophilic attack by OH-2 on C-1, with elimination of ficaprenyl phosphate.

After 10 min, **4** was unchanged and **7** was 100% hydrolyzed. After 2 h and 4 h, **4** was hydrolyzed (10 and 25%, respectively) to yield ficaprenol as the only identifiable product.

Incorporation of D-[^{14}C]*glucose into endogenous acceptors.* — Incubation of the particulate fraction obtained from *Micrococcus lysodeikticus* with UDP-D-[^{14}C]glucose, and separation of the products according to their solubility, as described in the Experimental section, resulted in a rapid, initial incorporation of radioactivity into lipid products extracted with 2:1 chloroform–methanol (Fig. 1). This incorporation was linear for ~10 min, and then the proportion of glucolipid remained constant thereafter. In contrast, incorporation of radioactivity into the polymer-containing residue occurred at a lower rate, which remained nearly constant throughout the incubation period. The incorporation of radioactivity into products extracted with 10:10:3 chloroform–methanol–water was very low throughout the incubation.

There was no lag in incorporation into the polymer fraction, as had been observed previously[7,8]. The addition of UDP-ManNAcA and UDP-GlcNAc (0.01mM), alone or in combination, had no effect on the incorporation into any of

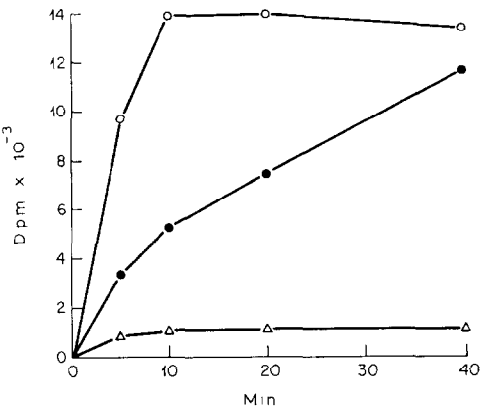


Fig. 1. Incorporation of radioactivity from UDP-D-[^{14}C]glucose into endogenous acceptors. Incubation was performed under standard conditions (pH 7.8) for different periods of time (see Experimental section). Radioactivity in the chloroform–methanol extract (○——○), in the chloroform–methanol–water extract (△), and in the residue (●——●).

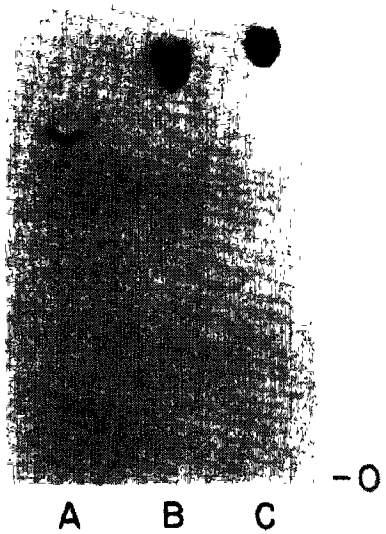


Fig 2 T.l.c on silica gel of chloroform–methanol extract labeled from UDP-D-[^{14}C]glucose (A) Chloroform–methanol extract; (B) synthetic ficaprenyl β-D-glucopyranosyl phosphate (7), (C) synthetic ficaprenyl α-D-glucopyranosyl phosphate (4) T l.c was performed with two successive developments in solvent C The labeled compound was detected by radioautography, and the synthetic compounds with phosphomolybdic spray O, origin

the products. Furthermore, tumicamycin (100 μg/mL), in the absence or presence of the aforementioned nucleotide sugars, did not affect the extent of labeling. In these respects, the particulate enzyme preparation used in the present study differs greatly from those used by other workers[7–10]

The optimum pH for incorporation into the lipid fractions was 6.0–6.5 in Tris-maleate buffer, whereas a very broad pH optimum between 6.5 and 8.5 was observed for incorporation into the residue. Addition of magnesium chloride to the incubation mixture greatly stimulated incorporation into the residue. The maximum effect was observed at a concentration of 30mM magnesium chloride, which resulted in a four-fold stimulation. The addition of 5–40mM magnesium chloride stimulated incorporation of only ~20% into the chloroform–methanol extract. This small effect does not exclude a requirement for divalent cations for the synthesis of the lipid products, as sufficient magnesium chloride may have been

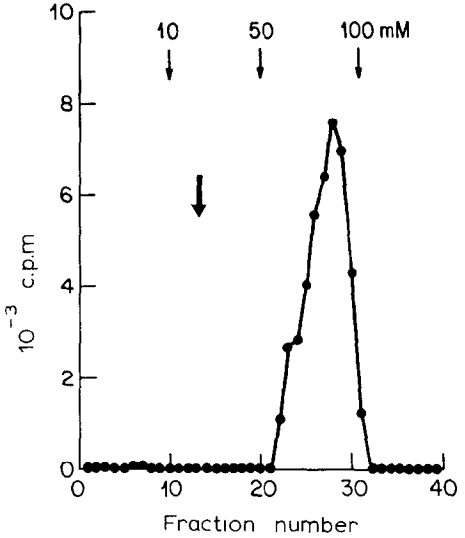


Fig. 3. Chromatography on DEAE-cellulose of [^{14}C]glucolipid. A chloroform–methanol extract obtained from incubation with UDP-D-[^{14}C]glucose under standard conditions was mixed with synthetic ficaprenyl α-D-glucopyranosyl phosphate (**4**) and chromatographed on a column (0.6 × 3 cm) of DEAE-cellulose (AcO^-), previously equilibrated with 10:10:3 chloroform–methanol–water. Sequential elution with 10:10:3 chloroform–methanol–10mM ammonium acetate, 10:10:3 chloroform–methanol–50mM aqueous ammonium acetate, and 10:10:3 chloroform–methanol–100mM aqueous ammonium acetate was performed, and fractions (1 mL) were collected for radioactivity measurements. Recovery of radioactivity was 80%. The arrow indicates the elution volume of synthetic ficaprenyl α-D-glucopyranosyl phosphate (**4**).

present in the particulate preparation washed with TMM buffer, which itself contained magnesium chloride.

Strong-acid hydrolysis of the labeled fractions obtained from UDP-D-[^{14}C]glucose, followed by paper chromatography in solvents *E* and *F*, showed that all the radioactivity incorporated into the chloroform–methanol extract, and into the residue, migrated as D-glucose.

Properties of [^{14}C]glucolipid in the chloroform–methanol extract. — The chloroform–methanol extract showed a single labeled product when examined by radioautography after t.l.c. in solvent *C*. The labeled glucolipid migrated more slowly than synthetic ficaprenyl α- (**4**) and β-D-glucopyranosyl phosphate (**7**) (Fig. 2). Upon chromatography of the chloroform–methanol extract on DEAE-cellulose, the labeled glucolipid was retained on the column and subsequently eluted with 50mM ammonium acetate (Fig. 3). Synthetic ficaprenyl (α-D-glucopyranosyl phosphate) (**4**) was eluted at a much lower concentration of ammonium acetate under the same conditions.

The labeled glucolipid was extremely labile under a variety of conditions. Stopping the incubation by boiling the mixture caused significant degradation, as did elution of the glucolipid from the thin-layer chromatograms and rechromatography. The half-life in 0.1M hydrochloric acid at 22° was ~1 min. The labeled prod-

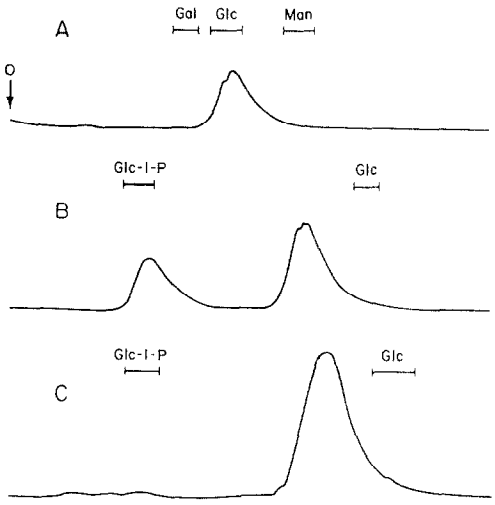


Fig 4 Paper chromatography of products of acid and alkali treament. (A) The chloroform–methanol extract obtained from a standard incubation of UDP-D-[^{14}C]glucose with *M lysodeikticus* particulate fraction was dried under nitrogen, and then treated with 0.01M hydrochloric acid in 50% aqueous 1-propanol for 15 min at 90°. The product was chromatographed in solvent *F* with standard sugars. (B) UDP-D-[^{14}C]glucose was treated with 2M ammonium hydroxide for 2 h at 37°. The product was chromatographed in solvent *G* with standard sugars. (C) The chloroform–methanol extract obtained from a standard incubation of UDP-D-[^{14}C]glucose with *M lysodeikticus* particulate fraction was dried under nitrogen, and then treated with 2M ammonium hydroxide in methanol for 2 h at 37°. The product was chromatographed in solvent *G* with standard sugars. The standard sugars were detected with the alkaline silver nitrate reagent, and the products by radioactivity. Glc-1-P, α-D-glucopyranosyl phosphate

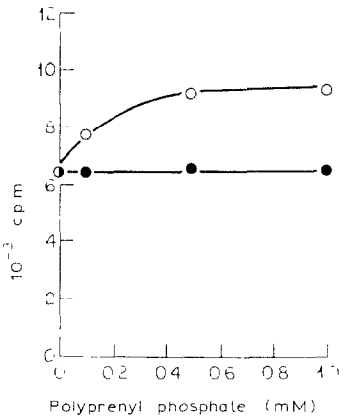


Fig. 5. Effect of exogenous polyprenyl phosphate on the incorporation of radioactivity into the chloroform–methanol extract. The conditions of incubation are described in the Experimental section, except that the particulate fraction was suspended in buffer and kept at 4° overnight before use (○——○) ficaprenyl phosphate; (●——●) C_{55}-dolichyl phosphate.

uct formed under these conditions migrated with D-glucose in solvent *F* (Fig. 4A). The labeled glucolipid was also extremely labile to mild alkali treatment. It was completely degraded to water-soluble, labeled products within 1 min by treatment with 0.1M sodium hydroxide at 22°. After alkali treatment with 2M ammonium hydroxide for 2 h at 37°, α-D-glucopyranose 1,2-phosphate was formed (Fig. 4B,C), whereas treatment with 0.1M sodium hydroxide at 22° gave labeled D-glucose 2-phosphate and D-glucose. The labeled D-glucose 2-phosphate migrated slightly faster than α-D-glucopyranosyl phosphate and was resistant to mild acid treatment.

Effect of polyprenyl phosphates on glucolipid formation. — The addition of synthetic ficaprenyl phosphate, at a concentration of 0.5mM, to the incubation mixture stimulated by ~40% the incorporation of D-glucose into the lipid fraction (Fig. 5), whereas the addition of C_{55}-dolichyl phosphate had no effect. In order to observe this stimulation, the particulate fraction had to be suspended in buffer and kept overnight at 4° before incubation, presumably to allow some degradation of the endogenous lipid acceptor. The addition of ficaprenyl phosphate had no effect on the incorporation of radioactivity into the residue.

DISCUSSION

The present results demonstrate that particulate preparations of *M. lysodeikticus* incubated with UDP-D-[^{14}C]glucose synthesize a ^{14}C-labeled glucolipid that has the characteristic properties of a polyisoprenyl D-glucosyl diphosphate. This glucolipid is unusually labile under both mild acid and alkaline conditions. Its behavior on DEAE-cellulose, and the formation of α-D-glucopyranose 1,2-phosphate and D-glucose 2-phosphate by alkali treatment, are evidence for the presence of a pyrophosphoric ester linkage between sugar and lipid residues[22]. The observation that the labeled glucolipid migrates, on thin-layer chromatograms, with an R_F value lower than that of synthetic ficaprenyl glucosyl phosphates (**4** and **7**) also supports this conclusion. On the basis of earlier observations on the formation of polyisoprenyl glycosyl diphosphate[23], in which a glycosyl phosphate residue is transferred from the nucleotide sugar phosphate, it may be assumed that the sugar residue is present as an α-D-glucopyranosyl group. The polyisoprenyl nature of the lipid is suggested by the stimulation observed with ficaprenyl phosphate. This stimulation seems specific for the C_{55}-polyisoprenol phosphate having an unsaturated α-isoprene residue, as C_{55}-dolichyl phosphate, which has a saturated α-isoprene residue, had no effect. The effect of ficaprenyl phosphate was small, but it has been shown previously[24] that ficaprenyl phosphate (which has three internal, *trans* double-bonds) is not as efficient in the formation of lipid intermediates required for the formation of colominic acid in *Escherichia coli* as bacterial undecaprenyl phosphate (which has only two internal, *trans* double-bonds). The failure to detect this glucolipid in earlier studies [7–10] of *M. lysodeikticus* is probably because of its extreme instability, as, in those experiments, inactivation of incubation mixtures was performed under acidic conditions that would probably result in degrada-

tion. A similar glucolipid, which is probably involved in cellulose biosynthesis, has been described in *Acetobacter xylinum*[25]; and lipid intermediates containing oligosaccharides in which a D-glucose residue at the reducing end is linked to an undecaprenyl *via* a pyrophosphate group have been demonstrated in the biosynthesis of exopolysaccharides in certain strains of *Klebsiella aerogenes* and of *Escherichia coli*[23,26,27]. Although the function of the D-glucosyl polyisoprenyl diphosphate in the biosynthesis of cell-wall polymers of *M. lysodeikticus* remains to be determined, the results just discussed are strong evidence that, in this Gram-positive, as in all Gram-negative[28] micro-organisms, the synthesis of the antigenic polysaccharide proceeds through lipid intermediates.

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