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THE ATTACHMENT OF POLY(RIBITOL PHOSPHATE) TO LIPOTEICHOIC ACID CARRIER*

FRANZ FIEDLER† AND LUIS GLASER

Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University, Saint Louis, Missouri 63110 (U. S. A.)

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ABSTRACT

The linkage between the lipoteichoic acid carrier from *Staphylococcus aureus* and poly(ribitol phosphate) synthesized with poly(ribitol phosphate) polymerase has been shown to be a phosphoric diester bond between a glycerol residue in the lipoteichoic acid carrier molecule and a ribitol residue. The phosphate residue is derived from CDP-ribitol. Indirect evidence suggests that this glycerol residue is located at the hydrophobic end of the lipoteichoic acid carrier, which contains all the D-glucose and fatty acid residues of the molecule. Such an attachment site explains the requirement of poly(ribitol phosphate) polymerase for the intact lipoteichoic acid carrier molecule and its inabiiity to use poly(glycerol phosphate) as an acceptor.

INTRODUCTION

We have shown previously that a purified enzyme from *Staphylococcus aureus* catalyzes the synthesis of poly(ribitol phosphate) according to the overall equation:

$$\text{LTC}^{\ddagger} + 30\ (\text{CDP-ribitol}) \rightarrow \text{LTC-(ribitol phosphate)}_{30} + 30\ \text{CMP}$$

where LTC is the lipoteichoic acid carrier, a molecule that is identical to the lipoteichoic acid present in the membrane of this organism. LTC has the overall composition of 12–14 glycerol phosphate units, one D-glucose residue, and one fatty acid residue per mole. The evidence for the linkage of poly(ribitol phosphate) to LTC was the change in size of radioactive LTC after incubation with enzyme and CDP-ribitol as determined by polyacrylamide disc-gel electrophoresis[1,2].

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‡ The nonstandard abbreviation used is LTC: lipoteichoic acid carrier.

In this communication, we wish to examine the chemical nature of the linkage between LTC and poly(ribitol phosphate). An elucidation of the site of attachment of poly(ribitol phosphate) to LTC is a prerequisite for an understanding of the mechanism of this reaction. Two obvious sites for attachment of poly(ribitol phosphate) to LTC are at a hydroxyl group of a glycerol or of a D-glucose residue. We will show that poly(ribitol phosphate) is attached to a terminal glycerol residue of LTC by a phosphoric diester bond, and that the phosphate group in the linkage is derived from the phosphate group linked to the ribitol residue of CDP-ribitol. Indirect evidence suggests that the attachment site is probably located at the hydrophobic end of the LTC molecule.

EXPERIMENTAL

Materials. — LTC and purified poly(ribitol phosphate) were prepared as described previously[1,2]. D-[^{32}P]Ribose 5-phosphate was prepared by acid hydrolysis (M hydrochloric acid, 30 min, 100°) of [α-^{32}P]-labelled ATP (Ado-5′[^{32}P]PP) (Amersham/Searle Corp., Arlington Heights, Ill. 60005) and was converted into [^{32}P]CDP-ribitol (5×10^7 c.p.m./μmole) by published procedures[1,3]. *sn*-[^{14}C]-Glycerol 3-phosphate was obtained from New England Nuclear (Boston, Mass. 02118). The following enzymes were obtained from Sigma (Chemical Co. St. Louis, Mo. 63178): *sn*-glycerol 3-phosphate dehydrogenase (rabbit muscle) (EC 1.1.99.5), triose phosphate isomerase (baker's yeast) (EC 5.3.1.1), and phosphoglycerate mutase (rabbit muscle) (EC 5.4.2.1). Crystalline D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.13) was a gift of Drs. C. Furfine and C. Frieden. NAD and D-glyceric acid 2,3-diphosphate were obtained from Sigma.

Methods. — Radioactivity was determined with a Packard liquid scintillation counter with 3a70 (Research Products International Corp., Elk Grove Village, Ill. 60007) as scintillation fluid. Gas–liquid chromatography of phosphate esters was performed on a 6-ft, 3% SE-30 column on 100–120 Varipore 30 in a Varian gas chromatograph equipped with a stream splitter. The phosphate esters were converted into free acids by passage through short columns of Dowex-50 (H^+), dried under vacuum, and converted into trimethylsilyl derivatives with 1:1:0.2 (v/v) pyridine–trifluorobis(trimethylsilyl)acetamide–chlorotrimethylsilane. The retention times at 165° for a carrier gas flow rate of 20 ml/min were: inorganic phosphate (1.3 min), 1,2-ethanediol phosphate (4 min), *sn*-glycerol 2-phosphate (10.3 min), and *sn*-glycerol 3-phosphate (12.4 min). Descending paper chromatography of sugars was performed on Whatman 3 MM paper with 6:4:3 (v/v) 1-butanol–pyridine–water as the solvent.

RESULTS AND DISCUSSION

The method for determining the linkage between poly(ribitol phosphate) and LTC is illustrated schematically in Fig. 1. LTC is loaded with ribitol [^{32}P]phosphate

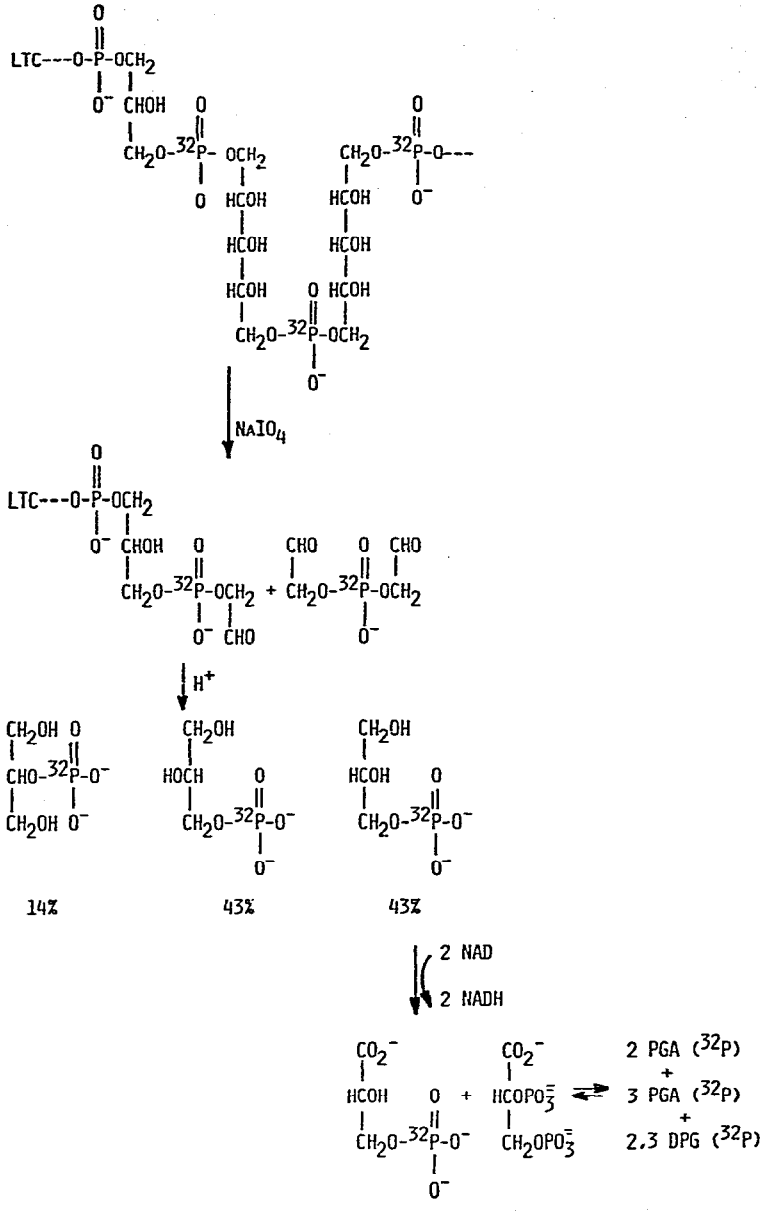


Fig. 1. Degradation of LTC-(ribitol [^{32}P]phosphate)$_n$. For details, see text. The representation of the terminal glycerol phosphate residue of LTC is purely diagramatic; as discussed in the text, it is likely to contain additional substituents. The ratio of *sn*-glycerol 2- and 3-phosphate residues was previously published[4] and is approximate. PGA, D-glyceric acid monophosphate; DPG, D-glyceric acid 2,3-bisphosphate.

by incubation with an excess of poly(ribitol phosphate) and [^{32}P]CDP-ribitol[1]. After periodate oxidation, if the linkage between poly(ribitol phosphate) and LTC is as shown in Fig. 1, acid hydrolysis should yield glycerol [^{32}P]phosphate where the phosphate group is derived from the terminal ribitol phosphate residue and the glycerol is derived from LTC. If the linkage between LTC and poly(ribitol phosphate) involves a terminal phosphate residue of LTC linked by a pyrophosphate bridge to poly(ribitol phosphate), no ^{32}P-labelled glycerol phosphate will be formed, nor will [^{32}P]glycerol phosphate be formed if the linkage between poly(ribitol phosphate) and LTC is a phosphoric diester bridge from ribitol phosphate to the D-glucose residues of LTC.

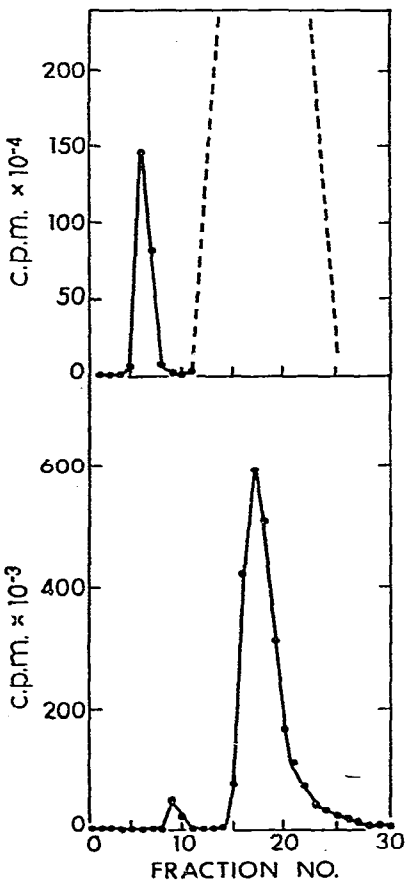


Fig. 2. Separation of LTC polymers from low-molecular-weight compounds. Top: Separation of LTC-(ribitol [^{32}P]phosphate)$_n$ from unreacted CDP-ribitol. LTC was incubated with [^{32}P]CDP-ribitol and poly(ribitol phosphate) polymerase[1]. At the end of incubation, a 1-ml sample was applied to a column (0.9 × 60 cm) of Bio Gel P-30 in 0.1% Triton and 1.5-ml fractions were collected. Bottom: The high-molecular-weight fraction from the preceding fractionation was oxidized with sodium metaperiodate, as described in the text, and chromatographed on a column (0.9 × 60 cm) of Sephadex G-25 in 0.1% Triton; 1.5-ml fractions were collected. Note that the columns used for the separations described in top and bottom figures were packed with different materials.

To test these possibilities, LTC was incubated with excess poly(ribitol phosphate) polymerase and [^{32}P]CDP-ribitol. The polymer was separated from the excess of nucleotide on a Bio-Gel P-30 column (Fig. 2, top). All the radioactivity in this fraction was shown to be associated with LTC-poly(ribitol phosphate) by polyacrylamide gel electrophoresis[2]. The polymer solution [0.1 μmole of LTC-poly(ribitol phosphate)] was lyophilized, and the residue was treated, at pH 6.0 with sodium metaperiodate (6 μmoles), in a final volume of 1 ml, for 18 h at 25° in the dark. Residual periodate was destroyed by the addition of excess 1,2-ethanediol, and the high-molecular-weight fraction was separated from the oxidation products by chromatography on a Sephadex G-25 column (Fig. 2, bottom). The high-molecular-weight fraction contained 3% of the original radioactivity (Table I) and should represent only the phosphate group at the linkage region (Fig. 1). This result is in excellent agreement with previous results in which the chain length of poly(ribitol phosphate) was determined by measuring the formation of [^{14}C]formaldehyde from LTC-[^{14}C]poly(ribitol phosphate) and found to be 30 units[2].

TABLE I

PRODUCTS OF DEGRADATION OF LTC-(RIBITOL [^{32}P]PHOSPHATE)$_n$[a]

Compounds	*Radioactivity*		
	^{14}C (*c.p.m.* × 10^4)	^{32}P (*c.p.m.*)	$^{14}C/^{32}P$
Initial LTC(ribitol phosphate)$_n$		2.6×10^6	
High-molecular-weight fraction after periodate oxidation		7.5×10^4	
Low-molecular-weight fraction after periodate oxidation		2.5×10^6	
Glycerol phosphate (Fig. 3A)	4	4.7×10^4	0.85
After enzymic oxidation (Fig. 3B):			
Residual glycerol phosphate	1.7	2.7×10^4	
Glyceric acid 3-phosphate	1.7	7.0×10^3	2.4
After phosphoglycerate mutase (Fig. 3C):			
Glyceric acid monophosphate	0.41	1.4×10^3	2.9
Glyceric acid 2,3-bisphosphate	0.93	4.6×10^3	2.1

[a]The data are from the samples described in the legends of Figs. 2 and 3, and have been corrected for ^{32}P decay. For details, see text.

The high-molecular-weight fraction from the Sephadex G-25 column was hydrolyzed in M hydrochloric acid for 3 h at 100°. It is expected that cleavage of the phosphate diester linkage between glycerol and glycolaldehyde (Fig. 1) would cleave preferentially to leave the phosphate group attached to glycerol. The acid hydrolysis conditions are drastic enough that other possible phosphate esters, such as D-glyceraldehyde 3-phosphate, formed by periodate oxidation of the D-glucose residue, to which poly(ribitol phosphate) was linked at C-6, would be totally hydrolyzed under these conditions[5].

The acid-hydrolyzed material was evaporated to dryness under vacuum and *sn*-[^{14}C]glycerol 3-phosphate (0.5 μmole, 40,000 c.p.m.) was added and the mixture chromatographed on a column (1 × 14 cm) of Dowex 1 X-8 (Cl^-) and eluted with 0.01M hydrochloric acid[6] (Fig. 3A). The major ^{32}P-labelled peak coincided in elution

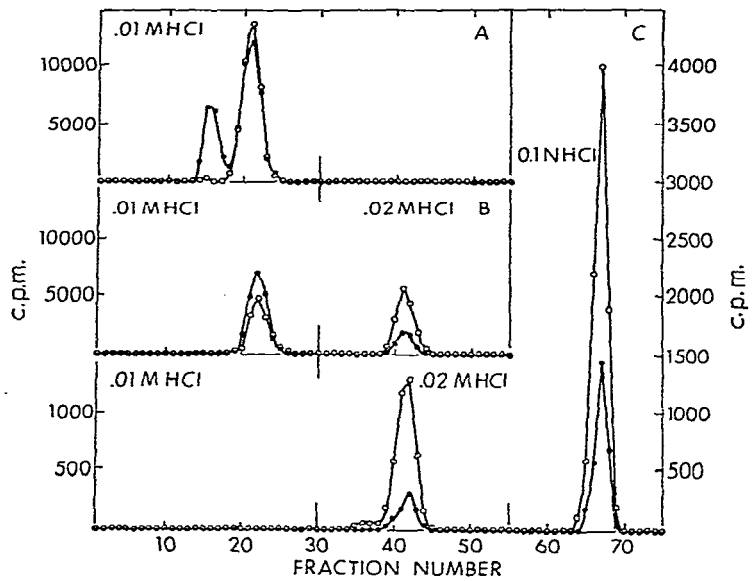
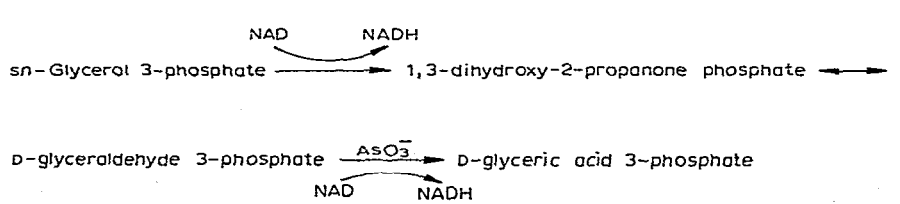


Fig. 3. Chromatography of degradation products of LTC. Chromatography was performed on a column (1 × 14 cm) of Dowex 1 X-8 (Cl^-), eluted with the solvents indicated, 10-ml fractions were collected: ● ^{32}P, O ^{14}C. (A) Glycerol phosphate after acid hydrolysis of periodate-oxidized polymer and addition of *sn*-[^{14}C]glycerol 3-phosphate. The identity of the small, initial peak, that contains ^{32}P but not ^{14}C is not known. (B) Reaction mixture after oxidation of *sn*-glycerol 3-phosphates to D-glyceric acid 3-phosphate. (C) Reaction mixture after equilibration of D-glyceric acid 3-phosphate with D-glyceric acid 2,3-bisphosphate. For details, see text.

position with added *sn*-glycerol 3-phosphate. The pooled sample in this peak was evaporated under vacuum and converted enzymically into D-glyceric acid 3-phosphate by reaction sequence (*1*);



(*1*)

Because of the unfavorable equilibrium constant of the two dehydrogenases, it is difficult to drive this reaction to completion. In addition, the glycerol [^{32}P]-phosphate obtained by acid hydrolysis should be a mixture of *sn*-glycerol 1- and

3-phosphate and *sn*-glycerol 2-phosphate (Fig. 1), and only *sn*-glycerol 3-phosphate will be converted into D-glyceric acid 3-phosphate.

The reaction mixture (1 ml) contained sodium carbonate (50 μmoles), sodium arsenate (30 μmoles), dithiothreitol (8 μmoles), NAD (0.4 μmoles), *sn*-glycerol 3-phosphate dehydrogenase (250 μg), triosephosphate isomerase (125 μg), D-glyceraldehyde 3-phosphate dehydrogenase (100 μg), and the mixture of glycerol phosphate eluted from the Dowex column (Fig. 3A). When the reaction stopped, as determined by the change in absorbance at 340 nm, the reaction mixture was placed on a column (1 × 14 cm) of Dowex 1 X-8 (Cl^-) ion-exchange resin and treated sequentially with 0.01M hydrochloric acid to elute unreacted glycerol phosphates, and with 0.02M hydrochloric acid to elute D-glyceric acid 3-phosphate (Fig. 3B). Since the glyceric acid [^{32}P]-phosphate is derived from a mixture of *sn*-glycerol 1- and 3-phosphate, whereas the [^{14}C]glycerol phosphate is pure *sn*-glycerol 3-phosphate, the ratio of [^{14}C] to [^{32}P] in glyceric acid 3-phosphate should be 3 times higher than in the original *sn*-glycerol 3-phosphate, which is essentially what is found in this experiment (Table I).

The identity of the glyceric acid 3-phosphate was further confirmed by incubation with phosphoglycerate mutase. This enzyme is known[7,8] to catalyze reaction (*2*).

D-Glyceric acid 3-[^{32}P]phosphate + D-glyceric acid 2,3-bisphosphate $\rightleftharpoons$
D-glyceric acid mono-[^{32}P]phosphate + D-glyceric acid 2,3-[^{32}P]bisphosphate (*2*)

Therefore, the ^{32}P- and ^{14}C-labelled D-glyceric acid phosphate, obtained as shown in Fig. 3B, was incubated in a final volume of 2.5 ml with tris(hydroxymethyl)aminomethane hydrochloride (100 μmoles), pH 7.5, magnesium chloride (10 μmoles), ethylenediamine tetraacetic acid (1 μmole), D-glyceric acid 3-phosphate (0.5 μmoles), D-glyceric acid 2,3-bisphosphate, and 2.5 units of phosphoglycerate mutase at 25° for 30 min. The reaction mixture was placed on a column (1 × 14 cm) of Dowex X-8 (Cl^-) ion-exchange resin and eluted successively with 0.01M, 0.02M, and 0.1M hydrochloric acid. D-Glyceric acid 2,3-bisphosphate was eluted with the last-named solvent and had the same ^{14}C to ^{32}P ratio as the original D-glyceric acid 3-phosphate.

A summary of the recoveries in the various steps is shown in Table I. The values have been corrected for ^{32}P decay during the experiment. The data are entirely consistent with the scheme shown in Fig. 1, and demonstrate that poly(ribitol phosphate) is linked to LTC *via* a phosphoric diester bridge between ribitol and a glycerol residue of LTC, and where the phosphate group is derived from the phosphate group linked to the ribitol residue of CDP-ribitol.

The high yield of glycerol phosphate obtained after acid hydrolysis of the periodate-oxidized polymer is only consistent with the linkage of poly(ribitol phosphate) by a phosphoric diester bridge to a terminal glycerol residue in LTC. Linkage to an internal glycerol residue would result in the formation of glycerol 1,2- or 1,3-bisphosphate, which is clearly not observed.

The structure of LTC is not known in detail, but current evidence would be consistent with a chain of poly(glycerol phosphate) units with a hydrophobic end containing fatty acid residues; the hydrophobic end also contains all the D-glucose residues in the molecule (Table II). Since D-glucose and fatty acids are the only possible substituents of glycerol in LTC, the hydrophilic end of the molecule

TABLE II

DISTRIBUTION OF D-GLUCOSE RESIDUES IN LTC[a]

Product	*D-Glucose (μmole)*	*Glycerol (μmole)*
Initial sample	0.12	1.7
Butanol phase after treatment with hydrofluoric acid	0.10	0.12

[a]LTC from *S. aureus* was degraded with 60% hydrofluoric acid to remove phosphate residues[9]. The hydrofluoric acid was removed under vacuum at 0° and the residue dissolved in water. The sample was extracted twice with butanol saturated with water (1 ml). The combined butanol phases were re-extracted with water and then evaporated and the residue dried under vacuum. The sample was hydrolyzed with M hydrochloric acid for 4 h at 100° and D-glucose and glycerol determined enzymically[10].

contains only unsubstituted glycerol residues, whereas the hydrophobic end contains a glycerol residue substituted either with fatty acids or with D-glucose residues. The ratio of D-glucose to glycerol in the hydrophobic region is equal to one. A terminal glycerol residue that is unsubstituted would, after periodate oxidation followed by borohydride reduction and acid hydrolysis, yield 1,2-ethanediol phosphate, whereas a substituted terminal glycerol residue would be resistant to periodate oxidation. Therefore, to test whether poly(ribitol phosphate) is attached at the unsubstituted glycerol residue at the hydrophilic end of LTC, [^{32}P]-LTC was loaded with unlabelled poly(ribitol phosphate) and isolated by chromatography on a Bio Gel P-30 column, similar to that described in the legend of Fig. 2. The material was totally converted into LTC-poly(ribitol phosphate) as judged by polyacrylamide gel electrophoresis[1].

This [^{32}P]LTC-(ribitol phosphate)$_n$ and, as a control, an equal sample of [^{32}P]LTC were oxidized with 15mM sodium metaperiodate in 0.05M sodium acetate, pH 5.0, for 4 h at 25° (the same results were obtained after longer periodate oxidation), excess periodate was destroyed by the addition of 60% glycerol (10 μl), and the sample dialyzed overnight against a large excess of water. The samples were reduced with sodium borohydride (2 mg) at 0° for 4 h, dried under vacuum, and hydrolyzed for 3 h at 100° in M hydrochloric acid, after addition of known quantities of 1,2-ethanediol phosphate and *sn*-glycerol 3-phosphate. After acid hydrolysis, the samples were dried under vacuum, dissolved in water (1 ml) and cations removed on a column (0.5 × 2 cm) of Dowex 50 X-8 (H^+) ion-exchange resin. The samples were dried under vacuum, borate ions were removed by evaporation with methanol, and the phosphate esters converted to trimethylsilyl derivatives as described under

"Methods". The ^{32}P content of 1,2 ethanediol phosphate and *sn*-glycerol 2- and 3-phosphates was determined by collecting samples from the gas chromatograph. The results are shown in Table III. Addition of poly(ribitol phosphate) had very little effect on the yield of 1,2-ethanediol phosphate from LTC*. This observation leads to the tentative conclusion that poly(ribitol phosphate) is attached to a substituted glycerol residue at the hydrophobic end of LTC.

TABLE III

YIELD OF 1,2-ETHANEDIOL PHOSPHATE FROM LTC AFTER PERIODATE OXIDATION AND SODIUM BOROHYDRIDE REDUCTION[a]

Degradation products	*Loaded LTC* ^{32}P *(c.p.m.)*	*Unloaded LTC* ^{32}P *(c.p.m.)*
sn-Glycerol 2- and 3-phosphates	10,300	11,700
1,2-Ethanediol phosphate	530	510

[a][^{32}P]LTC, either free or loaded with poly(ribitol phosphate) was oxidized with periodate and reduced with sodium borohydride. After acid hydrolysis, the yield of 1,2-ethanediol phosphate and glycerol phosphate was determined by g.l.c., as described in the text.

The attachment of poly(ribitol phosphate) to the hydrophobic end of LTC would explain the puzzling observation, made by us previously[1,2], that poly(glycerol phosphate), which lacks the hydrophobic end of the LTC molecule, cannot substitute for LTC in the poly(ribitol phosphate) polymerase reaction. Thus, the hydrophobic end of LTC would serve not only to anchor LTC to the membrane, but also would be the active site of the molecule.

It has previously been noted by Toon *et al.*[11] that the hydrophobic end of *S. faecalis* lipoteichoic acid contains glycerol, D-glucose, and fatty acid residues and the most likely structure is that of a phosphatidyl kojibiosyl diglyceride.

Whereas the conclusion that poly(ribitol phosphate) is linked to a terminal glycerol residue of LTC is quite clear, the assignment of this glycerol residue to the hydrophobic end of LTC is tentative, until a more precise structure of LTC can be determined.

ACKNOWLEDGMENTS

We are extremely grateful to Mrs. Barbara Lindsay for invaluable technical assistance, and to Dr. William Sherman for advice on the preparation of trimethylsilyl derivatives of phosphate esters.

*The yield of 1,2-ethanediol phosphate was approximately 5% of that of glycerol phosphate, which is consistent with a chain length of 12–14 glycerol phosphate units, one of which being converted to 1,2-ethanediol phosphate by periodate oxidation and borohydride reduction.

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