Biosynthesis of D-Alanyl-Lipoteichoic Acid in *Lactobacillus casei*: D-Alanyl-Lipophilic Compounds as Intermediates

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D-Alanyl-lipoteichoic acid (D-alanyl-LTA) from *Lactobacillus casei* contains a poly(glycerol phosphate) moiety that is selectively acylated with D-alanine ester residues. To characterize further the mechanism of D-alanine substitution, intermediates were sought that participate in the assembly of this LTA. From the incorporation system utilizing either toluene-treated cells or a combination of membrane fragments and supernatant fraction, a series of membrane-associated D-[14C]alanyl-lipophilic compounds was found. The assay of these compounds depended on their extractability into monophasic chloroform-methanol-water (0.8:3:2:1.0, vol/vol/vol) and subsequent partitioning into chloroform. Four lines of evidence suggested that the D-alanyl-lipophilic compounds are intermediates in the synthesis of D-alanyl-LTA. First, partial degradation of the poly(glycerol phosphate) moiety of D-alanyl-LTA by phosphodiesterase II/phosphatase from *Aspergillus niger* generated a series of D-alanyl-lipophilic compounds similar to those extracted from the toluene-treated cells during the incorporation of D-alanine. Second, enzymatic degradation of the D-alanyl-lipophilic compounds by the above procedure gave D-alanyl-glycerol, the same degradation product obtained from D-alanyl-LTA. Third, the incorporation of D-alanine into these compounds required the same components as the incorporation of D-alanine into membrane-associated D-alanyl-LTA. Fourth, the phosphate-induced loss of D-[14C]alanine-labeled lipophilic compounds could be correlated with the stimulation of phosphatidylglycerol synthesis in the presence of excess phosphate. We interpreted these experiments to indicate that the D-alanyl-lipophilic compounds are D-alanyl-LTA with short polymer chains and are most likely intermediates in the assembly of the completed polymer, D-alanyl-LTA.

The assembly of D-alanyl-lipoteichoic acid (D-alanyl-LTA) requires a biosynthetic system for selectively acylating the glycerol phosphate units of the poly(glycerol phosphate) moiety with D-alanine ester residues. These ester residues appear to have an important role in modulating the biological activity of this polymer. For example, Baddiley and co-workers (1, 20, 21, 26) have established that the capacity of this polymer to chelate Mg2+ is regulated in part by these ester residues. Recently, it was established by Fischer et al. (10, 11) that LTA containing D-alanyl residues did not function as an LTA carrier. Thus, the incorporation system for introducing these amino acid residues plays an important role in controlling the activity of D-alanyl-LTA.

In *Lactobacillus casei* the incorporation of D-alanine into LTA is accomplished in the following two-step reaction sequence (2, 27, 34, 36):

\[
\text{enzyme + D-alanine + ATP} \rightarrow \text{enzyme-AMP-D-alanine + PPi} \quad (1)
\]

\[
\text{enzyme-AMP-D-alanine + membrane acceptor} \rightarrow \text{D-alanyl-membrane acceptor + enzyme + AMP} \quad (2)
\]

In reaction 1, D-alanine is activated in the presence of ATP and the D-alanine activating enzyme to form an enzyme-AMP-D-alanine complex with the release of PPi. In reaction 2, the activated D-alanine is covalently linked to membrane acceptor in the presence of the D-alanine: membrane acceptor ligase. The precise function of the ligase has not been established.

The ultimate membrane acceptor in the reaction sequence has been established to be the poly(glycerol phosphate) moiety of D-alanyl-LTA (6). The isolation of D-[14C]alanyl-[3H]glycerol from D-[14C]alanyl-[3H]glycerol-labeled
d-alanyl-LTA supported this conclusion. The LTA from *L. casei* was found to contain either Glc(β1-6)Gal, (α1-2)acyl→6Glc(α1-3)diaicylglycerol or Glc(β1-6)Gal(α1-2)Glc(α1-3) diaicylglycerol as the glycolipid moiety (33). The poly(glycerol phosphate) moiety of this LTA is 37 to 44 units in length (33). To provide a better understanding of the mechanism of d-alanine incorporation, intermediates were sought that might participate in the assembly of d-alanyl-LTA. In 1977, Brautigan and Neuhaus (Fed. Proc. 36:931, 1977) described a series of membrane-associated lipophilic compounds which contained d-[14C]-alanine in ester linkage. It is the purpose of this communication to describe these compounds and implicate them as precursors in the assembly of d-alanyl-LTA.

**MATERIALS AND METHODS**

**Materials.** We are indebted to Eugene Kennedy for a generous gift of phosphodiesterase II from *Aspergillus niger* (38). d-[14C]alanine (40 mCi/mmol) and carrier-free P3 (32P) were purchased from the Amersham Corp. All phospholipids were obtained from Supelco and Applied Science Laboratories. Silica gel G-1 and silica gel G-25 HR thin-layer plates were purchased from Quantum Industries and Brinkmann Instruments Inc., respectively. Spectral-grade chloroform was purchased from Sargent-Welch. Wheat germ acid phosphatase was obtained from Worthington Biochemicals Corp. All other reagents were reagent grade. Membrane filters (pore size, 0.45 μm) were obtained from Millipore Corp. Sepharose 6B and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals, Inc.

**Preparation of toluene-treated cells, membranes, and supernatant fraction.** *L. casei* ATCC 7469 was grown according to the procedure described by Childs and Neuhaus (6). The toluene-treated cells were prepared as described previously (6). Membranes and supernatant fraction were prepared from *L. casei* by the procedures described by Linzer and Neuhaus (27) and Reusch and Neuhaus (36).

**Partial purification of d-alanine activating enzyme and d-alanine:membrane acceptor ligase.** These proteins have been partially purified from the supernatant fraction of *L. casei* by a modified procedure of Linzer and Neuhaus (27). Instead of resolving ligase and activating enzyme on Sephadex G-100, the filtration was accomplished on Sephacryl S-200 (2.5 by 100 cm). The column was equilibrated at 6°C with 50 mM piperazine acetate, pH 6.5, and eluted with the same buffer. The activating enzyme and the ligase were measured according to procedures described by Baddiley and Neuhaus (2) and Linzer and Neuhaus (27).

**Extraction assay for d-alanyl-lipophilic compounds.** The extraction assay measures the incorporation of d-[14C]alanine into chloroform-methanol-extractable-chloroform-soluble compounds. The reaction mixture contained the following components: 33 mM MgCl2, 5 mM ATP (adjusted to pH 6.5 with NaOH), 40 mM piperazine acetate (pH 6.5), 46 μM d-[14C]alanine (40 mCi/mmol), and either membranes and supernatant fraction or toluene-treated cells, in a total volume of 0.5 ml. The mixture was incubated at 37°C. For toluene-treated cells, 16 mg (wet weight)/0.5 ml was used. For membranes and supernatant fraction, 980 and 800 μg of protein respectively, were used.

The isolation of d-alanyl-lipophilic compounds was accomplished by the monophasic extraction procedure described by Bligh and Dyer (5). To terminate the reaction, 2 ml of extraction solvent (CHCl3-CH3OH, 1:0.38 [vol/vol]) was added to 0.5 ml of reaction mixture. This gave a monophasic system (CHCl3-CH3OH-H2O, 0.83:3:2.10 [vol/vol/vol]) which was mixed with a Vortex Genie mixer (Scientific Products) for 10 s and maintained at 37°C for 10 min. The extraction mixture was mixed again, and the sample mixture was filtered through a compact 0.6-cm glass wool plug in a 12.5-cm Pasteur pipette. Rinse solvent (0.5 ml) identical to the monophasic system was used to wash the residue.

The separation of d-alanyl-lipophilic compounds was accomplished by a modification of the procedure described by Bligh and Dyer (5). To the above filtrate (3.0 ml) 3.4 ml of separation solvent (CHCl3-CH3OH, 1:0.12 [vol/vol]) and 2.0 ml of 0.9% NaCl were added. The biphasic system was mixed vigorously and centrifuged for 10 min at one-quarter speed in an International clinical centrifuge to separate the organic and aqueous phases. The aqueous layer was removed, and an additional 2.0 ml of 0.9% NaCl was added. The biphasic system was mixed and centrifuged. The CHCl3 layer was transferred to a scintillation vial and evaporated, and 15 ml of scintillation fluid was added.

**Preparation of d-alanyl-LTA free of d-alanyl-lipophilic compounds.** d-[14C]alanyl-LTA was isolated from toluene-treated cells of *L. casei* as described by Childs and Neuhaus (6). After the d-[14C]alanyl-LTA was purified on Sepharose 6B (6), the sample was concentrated to 3 ml and the d-alanyl-lipophilic compounds were removed from the d-alanyl-LTA by the procedure described in the extraction assay. In the separation of phases, water was added in place of 0.9% NaCl. After the CHCl3 layer containing the d-alanyl-lipophilic compounds was removed, the aqueous phase with d-[14C]alanyl-LTA was concentrated to 4 ml. The concentrated d-[14C]alanyl-LTA was dialyzed against 50 mM formate buffer, pH 4.5.

**Alanine incorporation assay.** The alanine incorporation assay measures the incorporation of d-[14C]-alanine into either membranes or toluene-treated cells of *L. casei*, which were retained by a 0.45-μm-pore size membrane filter (6, 27, 36). This assay reflects incorporation into the d-alanyl-lipophilic compounds, d-alanyl-LTA, and activated d-alanine. The major component measured is d-alanyl-LTA.

**Chromatography of d-alanyl-lipophilic compounds.** For chromatography of the d-alanyl-lipophilic compounds, large reaction mixtures (2 ml), identical to those described for the extraction assay, were processed as described. The CHCl3 fraction containing the d-alanyl-lipophilic compounds was dried under N2. The residue was redissolved in CHCl3, and samples were applied to either silica gel Q-1 or silica gel G-25 HR (250 μm [20 by 20 cm]) thin-layer plates. The
plates were developed with a solvent consisting of butanol-acetic acid-water (120:30:50, vol/vol/vol). Autoradiograms were prepared by exposing Kodak XR-5 XOMAT X-ray film to the plate at -80°C.

**Analytical methods.** For the identification of phosphatidylglycerol (PG), the labeled phospholipid was compared with authentic standards by thin-layer chromatography in three solvent systems: (i) butanol-acetic acid-water (120:30:50, vol/vol/vol); (ii) CHCl₃-CH₂OH-acetic acid (65:15:13.5, vol/vol/vol); (iii) CHCl₃-CH₂OH-7 M NH₄OH (60:35.5, vol/vol/vol).

Protein was determined by the method of Lowry et al. (30), using bovine serum albumin as the standard. For the determination of radioactivity, the scintillation fluid described by Patterson and Greene (35) was used. Radioactivity was measured with a Packard model 2425 Tri-Carb liquid scintillation spectrometer.

**RESULTS**

Incorporation of d-[¹⁴C]alanine into d-alanyl-lipophilic compounds. A series of d-alanyl-lipophilic compounds was found which may participate in the assembly of d-alanyl-LTA. The assay of these lipophilic compounds depended on their extractability into monophasic chloroform-methanol-water (0.8:3.2:1.0; vol/vol/vol) and subsequent partitioning into the chloroform layer when the solvent was made biphasic. d-Alanyl-LTA was not soluble in the chloroform layer.

In Fig. 1, the incorporation of d-[¹⁴C]alanine into d-alanyl-lipophilic compounds is illustrated and compared with the incorporation of d-[¹⁴C]alanine into toluene-treated cells (Fig. 1A) and membranes (Fig. 1B). The toluene-treated cells provided a system with higher activity, whereas the combination of membrane fragments and supernatant fraction allowed one to establish the requirements for incorporation. The final membrane acceptor of the d-[¹⁴C]alanine has been identified as d-alanyl-LTA (6). In addition to d-alanyl-LTA, d-[¹⁴C]alanine incorporation into toluene-treated cells or membranes included d-alanyl-lipophilic compounds and, in the case of toluene-treated cells, activated d-alanine.

**Requirements for incorporation of d-[¹⁴C]alanine into d-alanyl-lipophilic compounds.** The incorporation of d-[¹⁴C]alanine into d-alanyl-LTA requires the d-alanine activating enzyme and the d-alanine:membrane acceptor ligase. These components have been partially purified from extracts of *L. casei* (27, 36). Incorporation into d-alanyl-lipophilic compounds is dependent on supernatant fraction, membranes, and ATP and is stimulated by Mg²⁺ (Table 1). The supernatant fraction could not be replaced by either the activating enzyme or the ligase. Both components were required to achieve optimal activity. These requirements were essentially identical to those for incorpo-

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**FIG. 1. Time courses of incorporation of d-[¹⁴C]alanine into d-alanyl-lipophilic compounds (○) and into (A) toluene-treated cells (■) and (B) membranes (○). The alanine incorporation assay was used for d-[¹⁴C]alanine incorporation into toluene-treated cells or membranes as described in the text. The extraction assay was used for measuring d-[¹⁴C]alanine incorporation into d-alanyl-lipophilic compounds. In (A) the reaction mixture contained 22 mg (wet weight) of toluene-treated cells per ml. In (B) the reaction mixture contained membrane fragments (2 mg of protein per ml) and supernatant fraction (2 mg of protein per ml). For d-alanyl-lipophilic compounds, 500-µl samples were assayed; for d-alanine incorporation into cells and membranes, 50-µl samples were assayed. The values of incorporation for both assays are presented for 50-µl reaction mixtures.**
TABLE 1. Requirements for 3-D-[14C]alanine incorporation

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity (pmol/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Alanyl-lipo-philic compounds</td>
<td>Membrane incorporation</td>
</tr>
<tr>
<td>Complete</td>
<td>1.30</td>
</tr>
<tr>
<td>-ATP</td>
<td>0.07</td>
</tr>
<tr>
<td>-MgCl₂</td>
<td>0.60</td>
</tr>
<tr>
<td>-Supernatant fraction</td>
<td>0.13</td>
</tr>
<tr>
<td>-Ligase</td>
<td>0.12</td>
</tr>
<tr>
<td>-Activating enzyme</td>
<td>0.68</td>
</tr>
<tr>
<td>-Membrane fragments</td>
<td>0.0</td>
</tr>
<tr>
<td>Zero time</td>
<td>0.52</td>
</tr>
<tr>
<td>Membranes (70°C)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

a D-Alanine incorporation into d-alanyl-lipophilic compounds was measured in the extraction assay (see text). The complete reaction mixture contained 25 mM MgCl₂, 5 mM ATP, 40 μM d-[14C]alanine (40 nCi/μmol), supernatant fraction (1 mg of protein), 30 mM piperazine acetate buffer (pH 6.5), and membrane fragments (1 mg of protein) in a total volume of 0.5 ml. The "minus ligase" reaction mixture contained 65 μg of activating enzyme as a replacement for the supernatant fraction, whereas the "minus activating enzyme" reaction mixture contained 65 μg of ligase (see text). Addition of ligase and activating enzyme replaces the requirement for supernatant fraction. Membranes (70°C) were heat inactivated at 70°C for 5 min and maintained at 4°C until used. The mixtures were incubated at 57°C for 1 h. For measuring d-[14C]alanine incorporation into membranes, the alane incorporation assay described in the text was used.

For comparison, the values of incorporation for both assays are presented for 50-μl reaction mixtures.

In the butanol-acetic acid-water (120:30:50, vol/vol/vol) solvent system, these compounds had Rfs in the range of 0.20 to 0.36 (Fig. 3). The chromatographic characteristics of these compounds indicated that they were a closely related set. The intensities of the spots did not reflect the relative amounts of the compounds, but rather they reflected the amounts of D-[14C]alanine which were covalently linked to the D-alanyl-lipophilic compounds. This series was well resolved from PG, digalactosyl diglyceride, and phosphatidic acid as well as alanine and D-alanyl-LTA. The requirements for incorporation into these compounds were consistent with those summarized in Table 1 (see Fig. 3 also).

Childs and Neuhaus (6) and Fischer et al. (10, 11) established that phosphodiesterase II/phosphatase from A. niger degraded d-alanyl-LTA with the liberation of d-alanyl-glycerol. In the same manner, treatment of D-alanyl-lipophilic compounds with this combination of enzymes also released the degradation product D-alanyl-glycerol. Characterization of this product was accomplished by procedures described by Childs and Neuhaus (6).

If the D-alanyl-lipophilic compounds are d-alanyl-LTA with short chains of poly(glycerol phosphate), it may be possible to form chloroform-soluble compounds from D-alanyl-LTA by enzymatically degrading the longer poly(glycerol phosphate) chains to shorter chains. Partially purified D-[14C]alanine-LTA was resolved from D-[14C]alanil-lipophilic compounds by the procedure described above. This extracted sample of d-alanyl-LTA was incubated with phosphodiesterase II/phosphatase. Samples were removed and assayed for D-alanyl-lipophilic compounds. Chloroform-soluble D-[14C]alanil-lipophilic compounds appear as the poly(glycerol phosphate) chains of the D-alanyl-LTA are shortened (Fig. 4). The maximal amount of D-[14C]alanine covalently linked to lipophilic compounds was achieved with a 48- to 72-h incubation. In longer incubations, the amount of D-[14C]alanine linked to these compounds decreased as the chains of poly(glycerol phosphate) were further shortened. This enzymatically degraded D-[14C]alanine-LTA that became chloroform soluble was chromatographed as described in Fig. 5. Eight radioactive spots, similar to those shown in Fig. 3, were observed (lane A, Fig. 5). For comparison, D-alanyl-lipophilic compounds extracted from partially purified D-[14C]alanine-LTA are shown in lane B. In lane C, D-[14C]alanil-lipophilic compounds extracted from toluene-treated cells that had been incubated with D-[14C]alanine are also shown. The Rfs for the D-[14C]alanil-lipophilic compounds in lane A are similar to those in lanes B and C.
D-ALANYL-LTA BIOSYNTHESIS IN L. CASEI

Fig. 2. Optimization of D-[14C]alanine incorporation into D-alanyl-lipophilic compounds: effect of (A) D-alanine, (B) ATP, (C) pH, and (D) ligase. The extraction assay for D-alanyl-lipophilic compounds is described in the text. For (A and B), D-[14C]alanine and ATP were varied, respectively. For (C) the piperazine acetate buffer of the incorporation assay was replaced with 80 mM Tris-maleate at the indicated pH. In (A and B), membranes and supernatant fraction as described in the text were used. In (C) toluene-treated cells (32 mg [wet weight] / ml) were used. In (D) the reaction mixtures contained 130 µg of purified activating enzyme per ml and increasing amounts of ligase (micrograms per milliliter), with 980 µg of membrane protein per ml.

The relative amounts of D-[14C]alanine label were somewhat different between D-alanyl-lipophilic compounds isolated by degrading D-[14C]alanyl-LTA and those isolated from toluene-treated cells.

The data in this section strongly suggest that the D-alanyl-lipophilic compounds isolated from the D-alanine incorporation system are most likely D-alanyl-LTA with short chains of D-alanyl-poly(glycerol phosphate). Thus, D-alanyl-LTA with short chains is soluble in chloroform, whereas D-alanyl-LTA with longer chains is insoluble. The D-alanyl-lipophilic compounds account for not more than 5% of the total D-[14C]alanine label in the D-[14C]alanyl-LTA described by Childs and Neuhaus (6).

On the biosynthetic relationship between the D-alanyl-lipophilic compounds and D-alanyl-LTA. The D-alanyl-lipophilic compounds may be either precursors or degradation products of D-alanyl-LTA. Although the time courses of incorporation (Fig. 1A and B) suggest that these compounds may result from degradation of D-alanyl-LTA, this conclusion may not be appropriate if D-[14C]alanine is being incorporated simultaneously into both D-alanyl-lipophilic compounds and D-alanyl-LTA. A decrease in the D-[14C]alanine-label in toluene-treated cells was observed after 1 h (Fig. 1A). Much of this released D-[14C]alanine label chromatographs with deacylated or partially deacylated D-alanyl-LTA (6). In contrast, the D-alanyl-lipophilic compounds attained a constant amount after 80 min. Thus, the release of D-[14C]alanine label as fatty acid-deacylated D-[14C]alanyl-LTA does not appear to contribute to the pool of D-alanyl-lipophilic compounds.

A different approach for analyzing the relationship between D-alanyl-lipophilic compounds and D-alanyl-LTA was initiated. It has been recognized in a variety of gram-positive bacteria that phosphate limitation results in the cessation
of wall teichoic acid biosynthesis (see below). Since our toluene-treated cells of L. casei are phosphate limited, addition of phosphate might initiate elongation of both populations of polymer, i.e., d-alanyl-lipophilic compounds and d-alanyl-LTA.

Toluene-treated cells were allowed to incorporate d-[14C]alanine for 17 min (Fig. 6A). The labeled cells were centrifuged and suspended in new incubation medium containing a 20-fold increase in the concentration of unlabeled d-alanine. At 32 min, 100 mM potassium phosphate was added. There was a rapid decrease in the d-[14C]alanine label in d-alanyl-lipophilic compounds. The decrease was more than 50% in 13 min. In the absence of phosphate, the amount of d-[14C]alanine incorporated into these compounds continued at a slower rate in the presence of a 20-fold increase of unlabeled d-alanine and at essentially the same rate in the presence of d-[14C]alanine. Potassium chloride and potassium nitrate did not cause a decrease in label, and potassium phosphate did not affect either the extraction of the d-alanyl-lipophilic com-
In contrast, the extraction assay for d-alanyl-lipophilic compounds measures a single group of compounds defined by their solubility in chloroform. A phosphate-initiated event in this assay may cause a decrease in the solubility of the d-alanyl-lipophilic compounds in chloroform, causing them to partition in the aqueous phase. Phosphate-limited, toluene-treated cells may be deficient in that pool of PG which is required for the synthesis of LTA (see below). A stimulation of PG synthesis which can be compared with a decrease of d-alanyl-lipophilic compounds may provide a basis for suggesting that these compounds are elongated upon addition of phosphate to the toluene-treated cells. Elongation of the poly(glycerol phosphate) moiety of the d-alanyl-lipophilic compounds would result in a change of solubility from chloroform to the aqueous phase.

Fig. 4. Formation of d-[14C]alanyl-lipophilic compounds from d-[14C]alanyl-LTA by degradation with phosphodiesterase II/phosphatase. The reaction mixture contained: d-[14C]alanyl-LTA free of d-[14C]alanyl-lipophilic compounds (see text) (255,000 dpm), 50 mM formate buffer (pH 4.5), 1.05 mg of phosphodiesterase II, and 2.3 mg of wheat germ acid phosphatase in a total volume of 5.25 ml. The mixture was incubated at 37°C, and samples (750 µl) were removed at the indicated times. The sample was diluted to 1 ml, and 0.5-ml aliquots were extracted as described in the extraction assay. The CHCl₃ layers were combined and assayed for radioactivity as described in the text.

Fig. 5. Thin-layer chromatography of d-[14C]alanyl-lipophilic compounds formed by degradation of d-[14C]alanyl-LTA with phosphodiesterase II/phosphatase. The isolation procedure for these d-[14C]alanyl-lipophilic compounds from 6.75 ml of reaction mixture is described in the legend to Fig. 4. The same relative concentrations of phosphodiesterase II/phosphatase and d-[14C]alanyl-LTA free of d-[14C]alanyl-lipophilic compounds were used. The mixture was incubated for 60 h. The d-[14C]alanyl-lipophilic compounds were extracted and concentrated in 100 µl of CHCl₃ and applied to lane A. Controls without phosphodiesterase II/phosphatase showed no d-[14C]alanyl-lipophilic compounds. In lane A, d-[14C]alanyl-lipophilic compounds extracted from d-[14C]alanyl-LTA as described in the text are shown. In lane B, d-[14C]alanyl-lipophilic compounds extracted from toluene-treated cells are shown.
Toluene-treated cells were incubated with carrier-free $^{32}$P for 30 min (Fig. 7, lanes 1 to 3). After this time interval, 100 mM phosphate was added to the cells. Within 2 min, a large increase in the amount of $[^{32}P]_{PG}$ was observed (Fig. 7, lane 4). At 40 min (10 min in the presence of phosphate), $[^{32}P]_{PG}$ is the major chloroform-soluble $^{32}$P-labeled compound (Fig. 7, lane 6). Little or no effect was detected in the other $^{32}$P-labeled compounds (e.g., $R_{f}$ 0.42 and 0.62, lanes 4 to 7). As expected, the simultaneous addition of carrier-free $^{32}$P and 100 mM phosphate at 30 min greatly reduced the specific activity of the labeled phosphate (lanes 9 and 10); thus, the apparent incorporation into the chloroform-soluble compounds was greatly diminished. However, if the $^{32}$P was added 30 min before the addition of 100 mM phosphate (lanes 1 to 7), the specific activity of the $^{32}$P label was not reduced to the same extent. It appears that the $^{32}$P was converted to a $^{32}$P-labeled intermediate and that the addition of phosphate greatly stimulated the conversion of this intermediate to $[^{32}P]_{PG}$. The $[^{32}P]_{PG}$, which was synthesized in response to excess phosphate, cochromatographed with authentic PG in three solvent systems (see above).

Thus, the addition of phosphate stimulated either PG synthesis from a $^{32}$P-labeled intermediate or the exchange of the glycerolphosphate moiety of PG, resulting in the incorporation of label. This rapid response to phosphate addition is similar to the response observed with the phosphate-induced loss of d-$[^{14}C]$alanine label in the d-$[^{14}C]$alanine-lipophilic compounds.

**DISCUSSION**

Four lines of evidence suggested that the d-alanyl-lipophilic compounds were d-alanyl-LTA with short chains of poly(glycerol phosphate). First, partial degradation of the poly(glycerol phosphate) moiety of d-alanyl-LTA by phosphodiesterase II/phosphatase from *A. niger* generated a series of d-alanyl-lipophilic compounds similar to those extracted from the membrane after the incorporation of d-alanine. Second, enzymatic degradation of the d-alanyl-lipophilic compounds by this procedure yielded d-alanyl-glycerol, the same degradation product obtained from d-alanyl-LTA. Third, the incorporation of d-alanine into these compounds required the same components as the incorporation of d-alanine into membrane-associated d-alanyl-LTA.
Fourth, the phosphate-induced loss of D-[14C]-alanine-labeled lipophilic compounds could be correlated with the stimulation of PG synthesis in the presence of excess phosphate. We interpreted these experiments to indicate that the D-alanyl-lipophilic compounds (D-alanyl-LTA with short polymer chains) are metabolically related to D-alanyl-LTA with longer polymer chains and that they are most likely intermediates in the assembly of the completed polymer, D-alanyl-LTA.

Fischer and co-workers (10-14, 25) presented evidence for a series of glycerophosphoglycolipids which are considered to be intermediates in the assembly of LTA. For example, from *Streptococcus lactis* NCDO 712, a di(glycerol phosphate)acylkojibiosyl diacylglycerol was characterized which was proposed on structural arguments to have a relationship to LTA. They also proposed that the D-alanyl-lipophilic compounds described by Brautigan and Neuhaus (Fed. Proc. 36:931, 1977) may be lipophilic short-chain segments similar to the series of glycerophosphoglycolipids from *S. lactis* (25). Fischer et al. (10) isolated and characterized a series of short-chained compounds from LTA prepared from *Leuconostoc mesenteroides* by an enzymatic procedure similar to that described in Fig. 4. It appears likely that these short-chained compounds are similar to the D-alanyl-lipophilic compounds shown in Fig. 5. In addition to the chain length heterogeneity of the poly(glycerol phosphate) moiety of the D-alanyl-lipophilic compounds, part of the observed het-
ergogeneity may also result from the presence of either of two glycolipid moieties in the LTA from *L. casei* 7469 (33).

The d-alanyl-lipophilic compounds described in this report have different solubility characteristics from the intermediates (nascent LTA) described by Ganfield and Pieringer (16). Although both sets of compounds were extracted by chloroform-methanol-water, the d-alanyl-lipophilic compounds partitioned into chloroform, whereas the nascent LTA partitioned into saline. Thus, we concluded that the compounds described in this report were different from those reported by Ganfield and Pieringer and represented ones at an earlier stage of assembly. In a study of LTA synthesis in *Streptococcus sanguis*, Emdur and Chiu (9) found one chloroform-methanol-extractable fraction which they characterized as LTA. This LTA may be similar to the d-alanyl-lipophilic compounds.

The correlation of $^{32}$P incorporation into PG with the loss of d-[$^{14}$C]alanine-labeled lipophilic compounds in the presence of excess phosphate provided indirect evidence of a biosynthetic relationship between the d-alanyl-lipophilic compounds and the presumed donor, PG. It is now widely concluded that PG is the donor of the sn-glycerol 1-phosphate units to the growing chain of LTA (8, 9, 16, 17, 31). In a number of bacteria PG is present in two distinct pools (3, 4, 28, 29, 39, 40). In *Bacillus megaterium* one pool (PG$_1$) undergoes rapid turnover of the phosphate moiety, whereas the second pool (PG$_2$) exhibits metabolic stability in this group (29). It has been suggested that PG is the donor of sn-glycerol 1-phosphate units for LTA synthesis (28, 29). Thus, one of the goals in the present work was to seek a correlation between the synthesis of PG and the metabolism of the d-alanyl-lipophilic compounds.

It has also been recognized that gram-positive bacteria which are phosphate limited synthesize teichuronic acid in place of teichoic acid (7, 15, 18, 19, 22, 37). In several bacteria, phosphate limitation can be correlated with a decrease in either CDP-glycerol pyrophosphorylase (22, 37) or UDP-GlcNAc:undecaprenyl phosphate phospho-GlcNAc transferase (19) and the synthesis of a protein(s) that inactivates one or more of the biosynthetic enzymes (18, 19). In *B. subtilis*, phosphate-limited cells synthesize only 10% of their phospholipid as PG, whereas magnesium-limited cells synthesize 54% of their phospholipid as PG (32). LTA synthesis has been reported to be unaffected by phosphate limitation in *B. subtilis* (D. C. Ellwood and D. W. Tempest, Proc. Biochem. Soc. 108:40P, 1968). However, in *B. licheniformis*, Button (D. Button, M. K. Choudhry, and N. L. Hemmings, Proc. Soc. Gen. Microbiol. 2:45, 1975) reported a 10-fold decrease in LTA in phosphate-limited cells when compared with magnesium-limited cells. These results together with the demonstration that one of the PG pools (PG$_3$) might be the donor of the sn-glycerol 1-phosphate units prompted us to examine the effect of excess phosphate on the metabolism of d-alanyl-lipophilic compounds and the synthesis of PG in toluene-treated cells.

In our experiments, a significant loss of d-[$^{14}$C]alanine-lipophilic compounds was observed when excess phosphate was added to the toluene-treated cells which had been labeled with d-alanine (Fig. 6A). In a separate series of experiments (Fig. 7), $^{32}$P$_2$ appeared to be incorporated into an intermediate which was subsequently converted to PG when excess phosphate was added to the $^{32}$P$_2$-labeled cells. Our interpretation of these results is that toluene-treated cells synthesize a unique population of PG in the presence of excess phosphate. This PG could donate sn-glycerol 1-phosphate units to the preexisting pool of d-alanyl-lipophilic compounds, resulting in their loss of chloroform solubility due to chain elongation. At the same time, it was presumed that sn-glycerol 1-phosphate from PG was transferred to glycolipid to generate new lipophilic compounds which could accept d-[$^{14}$C]alanine. Because of the turnover of the d-alanyl-lipophilic compounds and their relatively short time of exposure to the d-alanine incorporation system, the newly synthesized d-alanyl-lipophilic compounds would not become as extensively acylated with d-alanine as the preexisting d-alanyl-lipophilic compounds. Thus, addition of phosphate (Fig. 6A) caused a rapid loss of d-[$^{14}$C]alanine-labeled lipophilic compounds, whereas PG synthesis was stimulated by the addition of phosphate. It was interesting to note that the amount of d-[$^{14}$C]alanine incorporated into d-alanyl-lipophilic compounds began to increase 13 min after phosphate addition, even though the d-[$^{14}$C]alanine in the reaction mixture was removed and replaced with a 20-fold increase of unlabeled d-alanine. It is suggested that a fraction of the d-[$^{14}$C]alanine was retained by the toluene-treated cells as activated d-[$^{14}$C]alanine (adenyl-d-[$^{14}$C]alanine) bound to the d-alanine activating enzyme. As the turnover of d-alanyl-lipophilic compounds decreased, the existing compounds continued to accept activated d-[$^{14}$C]alanine, resulting in a low rate of net d-[$^{14}$C]alanine incorporation.

Phosphate-limited, toluene-treated cells appeared to be deficient in that PG which is required for elongation of the d-alanyl-lipophilic compounds. Thus, the d-alanine incorporation system continued to label the preexisting pool of d-alanyl-lipophilic compounds with d-[$^{14}$C]ala-
nine. Because of the deficiency in PG, we proposed that these lipophilic compounds could not be converted to the longer-chained chloroform-insoluble D-alanyl-LTA.

A possible intermediate in the D-alanine incorporation system is D-alanyl-PG. This phospholipid has been identified in Acholeplasma laidlawi (24) and Pediococcus cerevisiae (23); however, it has not been detected in L. casei (23). Attempts to demonstrate the synthesis of D-alanyl-PG in L. casei in our laboratory have been unsuccessful. Thus, it would appear that this phospholipid is probably not an intermediate in the synthesis of D-alanyl-LTA.

The experiments described in this report suggest that the D-alanyl-lipophilic compounds in the D-alanine incorporation system are D-alanyl-LTA with short chains of poly(glycerol phosphate). These short-chained compounds are most likely precursors of the longer-chained D-alanyl-LTA. Further study of the mechanism of synthesis of these compounds may provide clues to the sequence of reactions involved in the assembly of D-alanyl-LTA.

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