Effect of 3,4-Dihydroxybutyl-1-phosphonate on Phosphoglyceride and Lipoteichoic Acid Synthesis in Bacillus subtilis*

Robert M. Deutsch, Robert Engel, and Burton E. Tropp

From the Department of Chemistry, Queens College, City University of New York, Flushing, New York 11367

3,4-Dihydroxybutyl-1-phosphonate (CH₂OHCHOH-
\[\text{H}_2\text{CH}_2\text{PO}_3\text{HPO}_3\text{H}^\text{~} \]), an analogue of glycerol 3-phosphate, preferentially inhibits the rate of synthesis and accumulation of phosphatidylglycerol in Bacillus subtilis W23 and 168. The rate of phosphatidylethanolamine synthesis is only slightly inhibited, whereas that of lysylphosphatidylglycerol is somewhat stimulated. As expected, decreased phosphatidylglycerol synthesis results in the inhibition of the formation of the putative lipoteichoic acid precursor, sn-glycerol-1-phospho-β-gentiobiosyldiacylglycerol and of lipoteichoic acid itself.

3,4-Dihydroxybutyl-1-phosphonate, an analogue of glycerol 3-phosphate, is an inhibitor of phosphatidylglycerol synthesis (1). As such, it should affect the synthesis of those substances for which phosphatidylglycerol is a precursor. There is evidence suggesting that lipoteichoic is such a substance (2–5). The glycerol 3-phosphate monomer units in the polyglycerophosphate chain of lipoteichoic acid appear to be derived from the distal sn-glycerol 1-phosphate of phosphatidylglycerol (2). The chemistry and biochemistry of this membrane-associated amphipathic molecule of Gram-positive bacteria has been reviewed extensively (6–9). Fischer and co-workers have isolated and identified several glycerophosphoglycolipids that may be lipoteichoic acid precursors (10, 11). The precursor-product relationship between glycolipids and lipoteichoic acids was supported in studies of a mutant of Bacillus licheniformis lacking phospholipomutase (12). This mutant has a normal phosphoglyceride composition, but lacks the glycolipid gentiobiosyl diglyceride which is present in the wild type parent (12). The lipoteichoic acid isolated from the mutant lacks the gentiobiosyl diglyceride moiety normally attached to the polyglycerophosphate chain of the lipoteichoic acid of B. licheniformis (12).

To date, little is known about the effect of DHBP1 on Gram-positive microorganisms since most of the initial effort has been directed towards understanding its effects on the metabolism of Escherichia coli (13–17). At 2.5 mM, the drug is bacteriostatic to Bacillus subtilis 168 and E. coli and bactericidal to B. subtilis W23 (17, 18). These bacteria accumulate (1,2-diacyl)-sn-glycerol-3-phosphoryloxy-3′-hydroxybutyl-1′-phosphonate (the analogue of phosphatidylglycerophosphate) when treated with low concentrations of DHBP (13, 17). DHBP is a competitive substrate for the CDP-diacylglycerol: sn-glycerol 3-phosphate phosphatidyltransferase of E. coli (14). E. coli treated with 15 μM (5) DHBP exhibit an immediate inhibition of the rate of phosphatidylglycerol synthesis and a delayed but almost equally pronounced inhibition of the rate of phosphatidylethanolamine synthesis (1). The former effect has recently been exploited to support the hypothesis that phosphatidylglycerol is the source of the glycerol in the lipoprotein of E. coli (16). A similar rationale of approach is employed in the present study to demonstrate that the inhibition of phosphatidylglycerol synthesis in B. subtilis results in blocking the formation of the putative lipoteichoic acid precursor, sn-glycerol-1-phospho-β-gentiobiosyldiacylglycerol (10, 11) and in decreasing the rate of synthesis of lipoteichoic acid itself.

**MATERIALS AND METHODS**

**Chemicals—**rac-3,4-Dihydroxybutyl-1-phosphonate and rac-3,4-dihydroxy [3-2H]butyl-1-phosphonate were prepared as previously described (19). Tris, Sepharose 6B, Triton X-100 (octylphenoxypolyethoxyethanol), and casein hydrolysate were obtained from the Sigma Chemical Co., St. Louis, Mo. L-[^3]H]Leucine and [2-14C]glycerol were purchased from the New England Nuclear Corp., Boston, Mass. Carrier-free ([32P])phosphate was obtained from ICN Corp., Irvine, Calif. Anasul G thin layer plates were products of Analabs, Inc., New Haven, Conn. Bacterial phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin used as chromatographic standards were purchased from Supelco, Inc., Bellefonte, Pa. Kodak no-screen medical x-ray film, Kodak liquid x-ray developer, and Kodak rapid fixer were obtained from Eastman Kodak Co., New York, N.Y. All other chemicals were of reagent grade.

**Bacterial Strains and Culture Conditions—**B. subtilis BD 170 (thr*, trp*), a derivative of strain 168 (Marburg) and B. subtilis 1685 (met ), a derivative of strain W23 were kindly provided by Dr. L. Mindich and Dr. D. Dubnau of the Public Health Research Institute of the City of New York. The former contains polyglycerol phosphate and the latter polyribitol phosphate cell wall teichoic acid (30). The cells were cultured in 1% casein hydrolysate and 0.5% NaCl adjusted to pH 7 with NaOH at 37°C. Cell growth was monitored on the Klett-Summerson colorimeter as previously described (1, 17).

**Rate of Macromolecular and Phosphoglyceride Synthesis—** Cultures of B. subtilis at a turbidity of 20 Klett units were treated with the indicated concentration of rac-DHBP. The rate of DNA, RNA, and phosphoglyceride synthesis was determined by removing 1 ml of cells at the indicated times and incubating with 20 to 20 μCi of [32P]phosphate for 8 min. These samples were treated as previously described (1). The rate of protein synthesis was determined by removing 2 ml of cells at the indicated times and incubating with 2.5 μCi of [14C]leucine for 8 min. An equal volume of 10% trichloroacetic acid was added and the samples were kept at 0°C for 30 min prior to collecting, washing, and counting the precipitate by the method of Bollum as modified by Mans and Novelli (21).
The use of preconditioned medium prevented a lag in growth observed when log phase cultures are resuspended in fresh medium. The phosphoglyceride synthesis during recovery from drug treatment was determined as described above, except that the lipids were extracted directly from the cultures using chloroform and meth-
ane (1:2) without prior trichloroacetic acid treatment. The procedure for this isolation has been described (2). A parallel experiment performed with 60 μm rac-3,4-dihydroxyx[3-3P]butyl-1-phosphate (0.5 μCi/ml) indicated that most of the DHBP was removed after the cultures were washed and resuspended since no further incorporation into phospholipids was observed in the preconditioned medium lacking DHBP.

**Development of Phosphoglycerides-B. subtilis**

Recovery from DHBP Treatment—When the turbidity of a culture of **B. subtilis** reached 15 Klett units, 60 μm rac-DHBP was added. After a 35-min incubation, the cultures were filtered on Millipore filters (HA 0.45 μm), washed once with 5 ml of 0.1 M Tris buffer, pH 7.5 at 25°C, once with 5 ml of precondtioned casein hydrolysate medium, and then resuspended to a turbidity of 10 Klett units in precondtioned casein hydrolysate medium lacking DHBP-treated cultures had similar growth properties after resuspension. The rate of phosphoglyceride synthesis during recovery from drug treatment was determined as described above, except that the lipids were extracted directly from the cultures using chloroform and meth-
ane (1:2) without prior trichloroacetic acid treatment. The procedure for this isolation has been described (2). A parallel experiment performed with 60 μm rac-3,4-dihydroxyx[3-3P]butyl-1-phosphate (0.5 μCi/ml) indicated that most of the DHBP was removed after the cultures were washed and resuspended since no further incorporation into phospholipids was observed in the preconditioned medium lacking DHBP.

**Development of Phosphoglycerides-B. subtilis**

**Results and Discussion**

The effect of various concentrations of rac-DHBP upon the growth of **B. subtilis** W23 is depicted in Fig. 2. At 60 μm rac-
DHBP, the concentration at which most of the metabolic studies were performed, there is a slight inhibition of cell growth (Fig. 2). A similar effect upon the increase of the viable cell count was observed (data not shown). This concentration of drug caused a marked inhibition in the rate of phospholipid synthesis (approximately 30 to 35%) but had considerably less effect upon the rate of protein (approximately 3 to 5%), DNA (approximately 8 to 9%), and RNA (approximately 9 to 12%) synthesis. Analysis of the drugs' effect upon the individual phospholipids revealed a 20 to 30% inhibition in the rate of lysylphosphatidylglycerol synthesis, a 12 to 18% inhibition in the rate of phosphatidylethanolamine synthesis, and a 35 to 45% inhibition in the rate of phosphatidylglycerol synthesis (Fig. 3). In untreated cells, the rate of synthesis of phospha-
idylglycerol is approximately 4.5 times that of lysylphophatidylglycerol. On a molar basis, there is at least 7 times more inhibition in the rate of synthesis of phosphatidylglycerol than

**Fig. 1. Purification of lipoteichoic acid from **B. subtilis** 1005**

Downloaded from http://www.jbc.org/ by guest on September 22, 2017

1 Preconditioned medium was prepared by growing cells to 30 Klett units and resuspending them on Millipore filters. After centrifugation, the supernatant was used. The use of preconditioned medium prevented a lag in growth observed when log phase cultures are resuspended in fresh medium.

**Fig. 1. Purification of lipoteichoic acid from **B. subtilis** 1005**

Downloaded from http://www.jbc.org/ by guest on September 22, 2017

1 Preconditioned medium was prepared by growing cells to 30 Klett units and resuspending them on Millipore filters. After centrifugation, the supernatant was used. The use of preconditioned medium prevented a lag in growth observed when log phase cultures are resuspended in fresh medium.

**Fig. 1. Purification of lipoteichoic acid from **B. subtilis** 1005**

Downloaded from http://www.jbc.org/ by guest on September 22, 2017

1 Preconditioned medium was prepared by growing cells to 30 Klett units and resuspending them on Millipore filters. After centrifugation, the supernatant was used. The use of preconditioned medium prevented a lag in growth observed when log phase cultures are resuspended in fresh medium.
DHBP, An Inhibitor of Lipoteicholic Acid Synthesis

I

234

Hours

FIG. 2. Effect of DHBP upon the growth of B. subtilis 1005. The culture medium was 1% casein hydrolysate, pH 7.0, and 0.5% sodium chloride. Cells were cultured in 30 ml of medium in a 250-ml side arm flask and incubated in a water bath shaker set at 150 rpm and 37°C. Cell density was followed turbidimetrically in a Klett-Summerson colorimeter with a 660 nm filter. Symbols: O, untreated cells; ■, 30 µM DHBP; △, 60 µM DHBP; ●, 120 µM DHBP; □, 240 µM DHBP.

can be explained by the stimulation in the rate of synthesis of lysylphosphatidyglycerol.

The rate of turnover of prelabeled phospholipids was not affected by the presence of 60 µM rac-DHBP (data not shown). Therefore, one might expect drug-treated cells to exhibit a slight increase in the accumulation of lysylphosphatidyglycerol, a slight decrease in the accumulation of phosphatidylethanolamine, and a marked decrease in the accumulation of phosphatidylglycerol. This was in fact observed. Fig. 4 indicates the effect that 60 µM rac-DHBP had upon the accumulation of phosphatidylglycerol. The increase in lysylphosphatidylglycerol synthesis evident in rate and accumulation studies may reflect a regulatory process that is necessary to compensate for the acidic nature of the phosphatidylglycerophosphate analogue that accumulates under these conditions (30).

Two minor components of the phospholipid fraction were detected by thin layer chromatography. These two components are likely to be cardiolipin and glycophospholipids, respectively. Fischer et al. (11) identified sn-glycero-1-phospho-D-gentio-biosyl-diacylglycerol as the glycophospholipid present in B. subtilis (31). The second component had chromatographic properties that were identical to those of an authentic standard generously provided by Prof. Werner Fischer (Table I). As evident by comparing the data in Table I with that presented in Fig. 3, 60 µM rac-DHBP had an even greater inhibitory effect upon the rate of formation of the glycerophosphoglycolipid than it did upon that of phosphatidylglycerol.

Cells treated with 60 µM rac-DHBP have an abnormal phospholipid composition. It therefore seemed likely that there might be a mechanism to normalize the cellular phospholipid composition following the removal of drug. This hypothesis proved only partially correct.

Fig. 4. Accumulation of phosphatidylglycerol during treatment with DHBP in B. subtilis 1005. Cells were cultured in 30 ml of medium as described in Fig. 2. At a cell density of 10 Klett units, [32P]phosphate was added to the cultures. After a 1-h incubation with label, DHBP was added. Samples were removed at the indicated times and lipids were extracted. The amount of phosphatidylglycerol was determined as described under "Materials and Methods." Symbols: O, untreated; △, 60 µM DHBP.

DHBP, An Inhibitor of Lipoteicholic Acid Synthesis

1523

FIG. 3. Effect of DHBP upon the rate of synthesis of phosphatidylglycerol, lysylphosphatidylglycerol, and phosphatidylethanolamine of B. subtilis 1005. Phospholipids were extracted and chromatographed as described under "Materials and Methods." Individual spots, visualized by iodine vapors, were scraped into scintillation vials and counted. The counts were corrected for cell density. The values for the rate of synthesis of lysylphosphatidyglycerol, phosphatidylethanolamine, and phosphatidylglycerol for untreated cells at the 40-min time point are 825 cpm, 1,892 cpm, and 6,625 cpm/ml of culture, respectively. Symbols: ●, lysylphosphatidyglycerol; ■, phosphatidylethanolamine; △, phosphatidylglycerol.
DHBP, An Inhibitor of Lipoteichoic Acid Synthesis

Phospholipids were extracted and chromatographed as described under “Materials and Methods.” sn-Glycero-1-phospho-β-gentiobiosydiglycerol was identified by its migration properties on Silica Gel G developed in the following solvent systems: 1, chloroform:acetone:methanol:acetic acid:water (50:25:25:10:5, v/v); 2, chloroform:methanol:ammonia:water (50:35:5:2, v/v); 3, chloroform:acetone:methanol:acetic acid:water (50:20:10:10:5, v/v); and 4, chloroform:methanol:ammonia:water (65:25:15:4, v/v). In each case, the glycolipid migrated in the same fashion as authentic standard. The values in Solvent 1 and 2 are 0.67 and 0.51, respectively. Fischer et al. have provided the relevant information for Solvent 3 (6, 7). Solvent 4 involves multiple development and therefore no Rf value is reported.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Untreated</th>
<th>60 μM rac-DHBP</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoglycolipid</td>
<td>1,531</td>
<td>663</td>
<td>57</td>
</tr>
<tr>
<td>Total lipid</td>
<td>61,240</td>
<td>40,980</td>
<td>33</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>533</td>
<td>458</td>
<td>14</td>
</tr>
<tr>
<td>Total lipid</td>
<td>55,340</td>
<td>38,240</td>
<td>31</td>
</tr>
</tbody>
</table>

Fig. 5. Rate of synthesis of different phospholipid classes after removal of drug from B. subtilis 1005. When a culture reached 20 Klett units, DHBP was added to a final concentration of 60 μM. After 30 min, the drug was removed and the rate of lipid synthesis was determined as described under “Materials and Methods.” The data were treated as described in Fig. 3. Symbols: O, lysylphosphatidylglycerol; Δ, phosphatidylglycerol; ■, phosphatidylycerolphosphatidylethanolamine.

Fig. 6. The rate of protein, DNA, RNA, phospholipid, and lipoteichoic acid synthesis of B. subtilis 1005 as a function of DHBP concentration. At a cell density of 20 Klett units, varying amounts of drug were added to each culture. After 30 min, samples were removed from each culture and the rate of synthesis was determined as described under “Materials and Methods.” The determination of lipoteichoic acid synthesis is described in Fig. 1. The values for the rate of synthesis of protein, DNA, RNA, and phospholipid for untreated cells are 3,755 cpm, 4,563 cpm, 83,910 cpm, and 4,893 cpm/ml of culture, respectively. Symbols: O, protein; △, RNA; Δ, RNA; △, lipoteichoic acid; O, phospholipid.
Acid may play some of the same roles in disease mechanisms as have been proposed for lipopolysaccharide (36). The existence of a drug able to block lipoteichoic acid formation may have application to these and related questions.

Acknowledgments—We wish to thank Professor W. Fischer of the University of Erlangen-Nürnberg for his generous gift of sn-glycero-1-phospho-β-gentiobiosylacylglycerol. We also appreciate helpful discussions with Dr. R. A. Pieringer of Temple University.

REFERENCES

Effect of 3,4-dihydroxybutyl-1-phosphonate on phosphoglyceride and lipoteichoic acid synthesis in Bacillus subtilis.
R M Deutsch, R Engel and B E Tropp


Access the most updated version of this article at http://www.jbc.org/content/255/4/1521

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/255/4/1521.full.html#ref-list-1