Biochemical Studies of Bacterial Sporulation and Germination

XIV. Phospholipids in Bacillus megaterium

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The principal phospholipids of Bacillus megaterium throughout the cycle of growth and sporulation were found to be phosphatidylglycerol, diphasphatidylglycerol, phosphatidylethanolamine, and a hitherto unidentified isomer of glycosaminyl-phosphatidylglycerol. Phosphatidylglycerol predominated during vegetative cell growth and then declined as spores developed, whereas diphasphatidylglycerol became more prominent during spore maturation. The latter phospholipid was relatively inaccessible in the vegetative cell and was more accessible in the spore, as judged by solvent extraction under various conditions.

Extensive growth and elaboration of new membranes are important features in the development of the bacterial endospore. Until now, phospholipid analyses of sporeformers have been limited to the vegetative cellular phase. With respect to Bacillus megaterium, striking differences in composition, attributable to the strain and growth conditions, have been reported (28, 30). The studies of Op den Kamp et al. (20, 21; Ph.D. Thesis, Univ. of Utrecht, Utrecht, The Netherlands, 1968) have, in addition, identified a new phospholipid, a glucosaminyl derivative of phosphatidylglycerol (PG). We wished to know whether distinctive patterns in the nature and relative abundance of phospholipids might accompany the several stages in the cycle of differentiation and morphogenesis in this organism. We also wondered whether a sporulating cell might provide an opportunity to determine the heterogeneity of a bacterial cell’s membranes and related aspects of their composition, function, and metabolic fate.

In the present studies, we found that four compounds which comprise over 95% of the phospholipids are present throughout the growth and sporulation cycle. However, they show marked variations in relative and total amounts in relation to phases of growth and spore maturation. One of these compounds is a newly identified glucosaminyl derivative of PG, in which glucosamine is linked to the 1'-hydroxyl group of PG instead of the 2'-hydroxyl as in Op den Kamp's compound (Ph.D. Thesis, Univ. of Utrecht, Utrecht, The Netherlands, 1968). That the phospholipids are not uniformly distributed through the cell and spore is suggested by their differential extractability, especially that of diphasphatidylglycerol (DPG).

A companion report describes the levels of fatty acids and their distribution among the phosphatides and other lipids of B. megaterium during the growth and sporulation cycle (24).

MATERIALS AND METHODS

Growth of cells and spores. B. megaterium strain QM B1551, a gift from H. S. Levinson, was grown in a nutrient broth (Difco) supplemented with 0.1% glucose, 1 mM CaCl₂, 1 μM FeSO₄, 10 μM MnCl₂, 13 mM KCl, 1.6 mM MgSO₄, and 5 to 10 mc of ³²P inorganic phosphate (New England Nuclear Corp.) per liter. The concentration of phosphate in the nutrient broth was approximately 2 mM and was predominantly inorganic orthophosphate. The bacteria were grown in a culture volume one-twentieth of the flask volume, in flasks rotated rapidly at 37 C. The cells and spores were harvested without washing and, if not used directly, were stored as a paste at -10 C.

Extraction and separation of phospholipids. A weighed quantity of cell (or spor) paste was extracted in one-phase solvent as follows. Paste (e.g., 0.2 g) was suspended in sodium acetate buffer (pH 4.0; final concentration, 0.1 M) to a total volume of 1.0 ml (assuming the cell paste to be 80% water). Chloroform (1 ml) and methanol (2.2 ml) were then added and upon mixing gave a single phase. This solvent system (chloroform-methanol-water or buffer, 1:2:2:1) was adapted from Bligh and Dyer (2). After extraction for 1 hr at room temperature, the solids
were separated by centrifugation and were washed with 1 ml of one-phase solvent. The extract and wash were combined and treated with 0.25 volume each of chloroform and water. The lower phase (essentially all chloroform) represents the phospholipid extract.

Further extraction by sonic treatment of the residual solids (see above) or cell paste was carried out with 2 ml of one-phase solvent in the presence of 2 g of 120-μm glass beads with a Branson Sonifex in steel cups 1.8 cm in diameter. A salt-ice bath was used for cooling. Sonic treatment sufficient to disrupt cells and spores completely required from 5 to 25 pulses, 30 sec in duration, at a setting of 4 to 5 amp with a sonic probe 0.5 inch in diameter. All sonic treatments included the use of glass beads.

Phospholipids were separated and analyzed by thin-layer chromatography (TLC) with Stahl Silica Gel G plates (0.25 mm thick) and the following solvent systems (v/v): (i) chloroform-methanol-acetic acid-water (100:50:14:6); (ii) chloroform-methanol-concentrated ammonia (70:20:1.5); (iii) chloroform-methanol-water (65:25:4); (iv) diethyl ether-hexane (1:1); (v) chloroform-methanol-concentrated hydrochloric acid (87:13:0.5); oxalic acid-impregnated plates were used for solvent (v). Quantitative determinations were made by locating each phospholipid area with autoradiography, scraping the silica gel into a tube and extracting it with chloroform-methanol-water (10:5:1, v/v), and measuring the radioactivity of the extract.

Paper chromatography was carried out on silica-impregnated paper with diisobutylketone-acetic acid-water (8:5:1, v/v) as described by Marini et al. (18). Column chromatography on silicic acid was used for the large scale isolation of glucosaminylphosphatidylglycerol (GlcNPG).

The following compounds were used as standards: DPG synthesized as described by de Haas and van Deenen (11); PG, alanyl ester of PG (alaPG), and lysyl ester of PG (lysPG) synthesized as described by Bonsen et al. (3–5); phosphatidylethanolamine (PE) isolated from soya-lecithin as described by Scholfield and Dutton (25) and further purified by column chromatography; GlcNPG was kindly provided by J. A. F. Op den Kamp and was isolated from B. megaterium strain MK 10D: two isomers of glucosaminylglycerol were synthesized as described by Op den Kamp et al. (personal communication).

Hydrolysis and analysis of phospholipids. Alkaline hydrolysis of phospholipids was carried out according to Dawson et al. (9, 10). The hydrolysis products were chromatographed on Whatman no. 1 paper with propanol-concentrated ammonia-water (6:3:1, v/v). High-voltage electrophoresis (100 v/cm) was performed in 0.02 M citrate buffer (H⁺/Na⁺ = 70/30), and low-voltage electrophoresis (10 v/cm) was performed in pyridine-acetic acid-water (1:10:890, v/v), pH 3.6. Hydrolyses with phospholipases A, C, and D were carried out as described by Bonsen et al. (5).

Spot tests included: the molybdate reagent for phosphate according to Hanes and Isherwood (15); ninhydrin (0.5% in water-saturated n-butyl alcohol) for amino-containing compounds; the periodate-Schiff reagent as described by Baddiley et al. (1) for vicinal hydroxyl groups; iodine vapor for all lipids and 30% sulfuric acid for all compounds (only on TLC).

 Autoradiograms of radioactive chromatograms were made with Kodak X-ray film (blue-sensitive). Radioactivity was measured with an end-window gas-flow counter (Nuclear Chicago Corp.). The optical density of the cultures (A at 660 nm) was determined in cuvettes of 2-mm path length in a Zeiss spectrophotometer (PMQ II).

Phospholipid levels were based on specific radioactivity values determined by total phosphate analyses (7). Protein values were determined by the ninhydrin reaction on alkaline hydrolysates (8). Amino acid analysis carried out in a Spinco model 120B amino acid analyzer was based on the methods of Spackman, Stein, and Moore (26).

**RESULTS**

Identification of the phospholipids. Throughout growth and sporulation, four principal compounds were present. Their Rf values in TLC (solvent i) were generally about 0.95, 0.70, 0.65, and 0.50 (Fig. 1). These compounds, numbered in the order of their Rf values, were labeled as P by growth in 32P-medium and were purified by column chromatography on silica with mixtures of chloroform-methanol as eluants or by preparative TLC with solvent (i). In the latter case, the phospholipids were isolated from the silica by extraction with chloroform-methanol-water (10:5:1, v/v). The compounds were identified as follows.

**Compound 1.** This lipid was shown to be DPG. It had the same Rf value as DPG on paper chromatograms impregnated with silica and on TLC (solvents i, ii, and v). Mild alkaline hydrolysis gave a water-soluble phosphate ester with the same Rf value on paper chromatograms [solvent: n-propanol-concentrated ammonia-water (6:3:1, v/v)] and the same mobility in high-voltage electrophoresis as bis-(glycerylphosphoryl)glycerol (GPGP; obtained from synthetic DPG).

**Compound 2.** This fraction, which appeared to be the main phospholipid in all stages of the growth cycle, was identified as PG. This determination was based on Rf values on paper chromatograms and TLC (solvents i, ii, iii, and v), staining properties such as a positive reaction with the periodate-Schiff reagent, and chromatography and electrophoresis of an alkaline hydrolysis product. Synthetic PG was used as a reference compound.

**Compound 3.** This ninhydrin-positive phospholipid and its alkaline hydrolysis product had exactly the same properties as authentic PE and glycerophosphorylethanolamine (GPE), respectively.
 Compound 4. This compound was also ninhydrin-positive, but its chromatographic properties (on paper chromatogram and TLC) were different from the following amino-containing phospholipids: PE, lysPE, phosphatidylserine (PS), alaPG, lysPG, and GlcNPG isolated from B. megaterium strain MK 10 D (20). Further examination disclosed the following properties.

(i) Paper chromatography on silica-impregnated paper gave an $R_F$ value of 0.25 (cf. $R_F$ value for PE, 0.55). On TLC (solvent i) it ran just behind PE, whereas in solvent (iii) it had a much lower $R_F$ value than PE. It gave a positive reaction with the molybdate reagent. With the tricomplex staining method of Bungenberg de Jong (6), it gave a red color, indicating that it is a dipolar ion or a basic phospholipid. There was no reaction with the periodate-Schiff reagent.

(ii) Alkaline hydrolysis according to Dawson (9) and Dawson et al. (10) produced a distinct spot on paper chromatograms [solvent: n-propanol-concentrated ammonia-water (6:3:1, v/v)] with an $R_F$ value of 0.48 (cf. $R_F$ value for GPE, 0.54). On high-voltage electrophoresis the mobility was almost nil. This hydrolysis product was also ninhydrin-positive and stained well with the periodate-Schiff reagent.

(iii) Strong acid hydrolysis (6 N HCl for 18 hr at 110 C) gave glucosamine as the principal product. As determined on the amino acid analyzer, the compound was clearly separated from galactosamine. Acid hydrolyses performed for 4, 8, and 12 hr and analyzed for both glucosamine and phosphorus gave a ratio, by extrapolation, of 1:1.

(iv) Hydrolysis with phospholipase A from Crotalus adamanteus led to a complete conversion to a compound still positive with the molybdate and ninhydrin reagents, but with a lower $R_F$ value on TLC with solvent (i). This product was tentatively identified as a lysyl derivative of the original phospholipid.

(v) Hydrolysis with phospholipase C from B. cereus showed a complete conversion to a non-polar lipid (identical with a 1,2-diglyceride on TLC with solvent iv) and a water-soluble compound, which was molybdate- and ninhydrin-positive and was almost neutral on high-voltage electrophoresis.

(vi) Phospholipase D from green cabbage leaves failed to hydrolyze the compound.

(vii) Nitrous acid treatment of GlcNPG was known to convert it to PG (J. A. F. Op den Kamp, Ph.D. Thesis, Univ. of Utrecht, Utrecht, The Netherlands, 1968). The same transformation was found with this unknown phospholipid; there was a nearly quantitative formation of PG.

The foregoing findings established the structure to be a glucosamine derivative of PG. Differences in chromatographic properties with Op den Kamp's compound (Ph.D. Thesis, Univ. of Utrecht, Utrecht, The Netherlands, 1968) might then be attributed to an attachment of glucosamine to carbon 1 rather than carbon 2 of the glycerol or a difference in the glycosidic linkage, or both. Mild acidic hydrolysis (1 N HCl for 4 hr at 100 C) produced a mixture of ninhydrin-positive compounds on high-voltage electrophoresis. These compounds were compared with several reference compounds and were tentatively identified as glucosamine (about 5%), glucosaminylglycerol (about 60%), and an unidentified spot with very low mobility (about 35%), probably a mixture of glucosaminyl glycerophosphate and glucosaminylglycerophosphorylglycerol. The glucosaminylglycerol reacted with the periodate-Schiff reagent and is therefore identified as glucosaminyl-1-glycerol. In glucosaminylglycerol derived from the Op den Kamp compound, glucosamine is attached to the secondary hydroxyl group (Ph.D. Thesis, Univ. of Utrecht, Utrecht, The Nether-
lands, 1968) and is periodate-Schiff-negative. The type of glycosidic linkage has not been determined.

Phospholipid levels during growth and sporulation. The level of total phospholipids continued to increase even after the absorbance of the culture became stationary (Fig. 2, 3); it then began to decrease sharply at a stage when about half of the cells in the culture contained a refractile spore (Fig. 2, 3). This fall in phospholipids preceded the lysis that liberates free spores and occurred even earlier than the decrease in protein concentration which characterizes the later stages of sporulation. Since PG is the predominant phospholipid, the total values are largely a reflection of changes in the level of this component; the decrease in the level of PE, however, was even greater than that of PG. Among the other three principal phospholipids only DPG showed a different pattern. The level of DPG became constant when log-phase growth was completed, remained that way until the stage when most cells had refractile spores, and then increased to about three times that level by the time fully mature spores were produced.

It must be emphasized that these values, for example those of DPG, are not derived from a homogeneous population of cells, since it is apparent from Fig. 2 that the culture is not synchronous and that there is nearly a 2 hr span between the initiation and completion of a given stage in the sporulation process. Nor can it be assumed that the phospholipids are distributed uniformly throughout a given cell. The next section deals with this question.

Differential extraction of phospholipids. Up to the stage of refractile spores, from 85 to 90% of the total phospholipids were extracted by the one-phase solvent. Most remarkably, this extract contained very little DPG (Fig. 1B). Upon disruption of the cells by sonic treatment, the remainder of the phospholipids showed DPG to be a major if not the predominant compound (Fig. 1C). By the time sporangia contained mature spores, only half of the phospholipid was extracted directly and sonic disruption was required for extraction of the rest; there was no striking difference in pattern of the constituent phospholipids between the direct and sonic-treated extracts (Fig. 4).

Unlike the fluctuation in the levels of the total extractable phospholipids (Fig. 3), there appeared to be a relatively constant level of the not readily extractable portion in the case of each phospholipid (Fig. 4). With DPG, the large increase during spore maturation is almost entirely in the readily extracted fraction. The sharp losses in PG and PE during sporulation also seem to have been at the expense of the directly extracted material. The possible significance of this distinctive behavior with respect to solvent extraction is considered in the next two sections.

Extraction of DPG from vegetative cells. Microscopic examination, by phase contrast, of cells treated with the one-phase solvent showed them to have their intact bacillary shape with only a slight contraction from their original contours. The resistance of the DPG in these cells to direct extraction by the one-phase solvent may be due
to its linkage to compounds in the membranes which withstand the solvent or to an intracellular location of membranes inaccessible to the solvent. Disruption of the cell and membranes by sonic oscillation exposes the DPG to extraction but this result can be explained by either of these alternatives.

Extraction of log-phase cells in the presence of 10 mM ethylenediaminetetraacetic acid showed no marked improvement in DPG extraction and thus the likelihood of a strong metal chelation of DPG as the basis for its resistance to extraction is weakened. The use of 90% acetone, especially with ammonia, has been recommended for the extraction of mitochondrial phospholipids (13) including their DPG (W. Stoeckenius, personal communication). Log-phase cells exposed to the acetone solvents appeared emptied of their contents in most cases. The yield of phospholipids was lower, even after subsequent treatment in the solvent, probably as a result of the lower solubility in acetone of phospholipids with saturated fatty acids. In view of the apparent cellular disruption, these experiments permit no judgment as to the location of the DPG.

Treatment of log-phase cells with lysozyme (14), with or without protoplast formation, led to a ready solvent extraction of the DPG (Fig. 1D). Incubation of cell suspensions also resulted in considerable lysis and made the DPG extractable. These experiments were complicated by a twofold or even greater increase in the total level of DPG during the lytic procedure. Fluctuation in the level of DPG, especially as a function of cell treatment between harvesting and extraction, was difficult to control and assess. The extractability of DPG upon cellular lysis might be interpreted as the exposure of previously inaccessible membranes to the solvent. Nevertheless, the indications that there were extensive compositional changes in the phospholipids, within even the brief periods preceding cellular lysis, make such an interpretation uncertain.

Extraction of DPG from spores. The fact that sonic disruption is necessary to extract about half of the spore’s phospholipids (Fig. 4) may be taken to indicate that certain membranes in the intact spore are not accessible to the solvent. In this instance, unlike vegetative cells, this lack of extractability is not limited to DPG, but applies to all the phospholipids.

A distinctive location of DPG was found in the case of spores isolated from culture grown without glucose added to the medium. Such spores, unlike those in media supplemented with 0.1% glucose, had associated with them phospholipid which was readily removed by procedures commonly employed to prepare “clean” and viable spores. The predominant phospholipid removed by mild sonic treatment of an aqueous suspension was DPG (Table 1). Subsequent extraction of such spores by solvent without and then with sonic disruption revealed the phospholipid pattern observed in spores produced in glucose-supplemented cultures (compare Table 1 and Fig. 4). These observations might be ascribed to the presence of an exosporial membrane, presumably rich in DPG. An exosporial membrane is prominent in electron micrographs of B. cereus (14), but analysis of its composition (L. Motz and P. Gerhardt, Bacteriol. Proc., 1964, p. 14) did not specify phospholipids; a capsule-like
TABLE 1. Extraction of phospholipids from spores

<table>
<thead>
<tr>
<th>Prior treatment</th>
<th>DPG</th>
<th>PG</th>
<th>PE</th>
<th>GlcNPG</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>None..................</td>
<td>31</td>
<td>31</td>
<td>12</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>Mild sonic treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant fraction</td>
<td>70</td>
<td>8</td>
<td>7</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Pellet................</td>
<td>21</td>
<td>39</td>
<td>13</td>
<td>20</td>
<td>7</td>
</tr>
</tbody>
</table>

Spores (1.0 g) prepared in a medium without added glucose were first washed with cold 0.1 M NaCl and then were suspended in 0.1 M KCl to a volume of 4 ml. Part of this suspension was exposed to a mild sonic treatment (four 1-min pulses with a Mullard ultrasonic drill; no glass beads). The pellet, collected by centrifugation, was washed with 0.1 M NaCl (pellet); the supernatant fluid and wash were combined (supernatant fraction). Each sample (with or without this prior treatment) was completely extracted by a combination of direct solvent extraction and sonic treatment. The supernatant fraction contained 2.5 times as much phospholipid as the pellet.

exosporial structure has also been identified in certain strains of *B. megaterium* (27), although clear morphological evidence for an exosporium in this species has not been obtained. The absence of a DPG-rich membrane in the case of our glucose-supplemented cultures might be ascribed to a greater abundance of lytic enzymes which destroyed it during or subsequent to liberation of the spores. The phospholipid composition of cells during growth and sporulation in nutrient broth not supplemented with glucose was determined and appeared to be essentially similar to the pattern described here; DPG was similarly resistant to direct extraction by the single-phase solvent.

**DISCUSSION**

The presence of PG, PE, and DPG in *B. megaterium* is rather typical of the assortment of phospholipids found in bacteria (16). The glucosamine derivative of PG is, however, unusual, the only other examples of it having been found by Op den Kamp et al. (20, 21) in another strain of *B. megaterium* and by Phizackerley et al. (23) in *Pseudomonas ovalis*. In the latter case, no conclusive evidence was given for the exact structure. Op den Kamp et al. (20–22) found their glucosaminyl compound only when the pH of the growth medium was reduced to about 5; at neutral pH almost none of this compound was present. In our studies, however, GlcNPG was found under normal growth conditions. Whereas in Op den Kamp's strain (Ph.D. Thesis, Univ. of Utrecht, Utrecht, The Netherlands, 1968) about 20% of the phospholipids consisted of lysPG, our strain showed little, if any, amino acyl esters of PG. The apparent structure of our glucosamine compound is shown in Fig. 5.

After the completion of this manuscript, a report by P. J. R. Phizackerley and J. C. MacDougall (Abstr. Sharnbrook Meeting, 19 October 1968, Biochem. J. 110:57) appeared which describes a similar GlcNPG isomer in a strain of *B. megaterium*, with the further information that the glycosidic bond has the B configuration. The fact that the glucosamine is bound to the 1'-hydroxy group of PG (glycosidic link unspecified in our compound) and to the 2'-hydroxy group in Op den Kamp’s compound indicates that a search for an enzymatic biosynthetic activity [comparable to aminoacyl PG synthetase (17)] may disclose distinctive pathways for the formation of these two phosphatides.

There were no drastic changes in the phospholipid pattern as spores developed, nor were there any novel phospholipids to distinguish the spore from the vegetative cell. Thus, our findings in *B. megaterium* confirm essentially similar conclusions reached by Matches et al. (19) in their studies of *B. polymyxa*. The requirement for disruption of the spore in order to extract the phospholipids completely was also observed earlier in *B. megaterium* by Fitz-James (12) and by Yamakawa et al. (29). However, the differential extraction of phospholipids, particularly DPG, suggests that the phospholipids may be deployed in special ways in both the vegetative cells and spores. DPG, unlike the other phospholipids, was extractable from vegetative cells only after mechanical disruption or osmotic lysis. One possibility is that DPG is localized in intra-cellular structures, of which the mesosome is a speculative example. Our results also suggest that DPG is associated with structures that adhere to spores and are readily removed from them. Whether these external structures are exo-
sporal membranes is not certain. If they are adherent maternal cell material, then they are distinctive by virtue of their preponderant DPG composition.

Although our studies suggest a heterogeneity among the membranes of the growing, sporulating cell and the mature spore, we were unable, with available techniques, to achieve significant fractionations as judged by phospholipid analysis. Perhaps enzyme activities related to the growth or function of membranes may provide better guides for such fractionation studies in the future.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Recent electron microscopic studies of spores of many species of *Bacillus*, including this QM B1551 strain of *B. megaterium*, by S. C. Holt of the University of Massachusetts and E. Leadbetter of Amherst College have led them to regard the relatively unstructured, outermost surface layer of strain QM B1551 to be an exosporium comparable to the loose-fitting, outer layer of *B. cereus* (personal communication).

LITERATURE CITED