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Phospholipids of *Streptococcus faecalis*

J. M. DOS SANTOS MOTA,[1] J. A. F. OP DEN KAMP, H. M. VERHEIJ, AND L. L. M. VAN DEENEN

Laboratory of Biochemistry, Rijksuniversiteit te Utrecht, Vondellaan 26, Utrecht, The Netherlands

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Autoradiograms of total lipid extracts from *Streptococcus faecalis* ATCC 9790, harvested in the stationary phase from a medium containing ^{32}P-orthophosphate, showed six major spots. The corresponding compounds were identified as diphosphatidylglycerol (possibly with a penta acyl structure); phosphatidylglycerol; a provisionally identified mixture of alanylphosphatidylglycerol and of the 2'-lysyl-derivative of phosphatidylglycerol; the 3'-lysyl-derivative of phosphatidylglycerol, probably together with some arginylphosphatidylglycerol; a diglucosyl derivative of phosphatidylglycerol; and a compound which was tentatively identified as the 2',3'-dilysyl derivative of phosphatidylglycerol.

Data on the phospholipid composition of *Streptococcus faecalis* appear to be rather incomplete. Ikawa (22) reported that 41% of the "free" lipids corresponded to phospholipids, his results suggesting the presence of the lysylderivative of phosphatidylglycerol. Kolb et al. (27) found that practically all the lipid phosphorus was located in the cell membrane, the cell wall being devoid of phospholipids. Shockman et al. (41) suggested phosphatidic acid to be the major phospholipid of the membrane fraction, whereas Ibbott and Abrams (21) detected two polar lipids in the total lipid extract, the major one possibly corresponding to a derivative of diphosphatidylglycerol, the other one giving a positive reaction for carbohydrates. Vorbeck and Marinetti (50) reported for bacteria, harvested in the exponential phase of growth, a more complex phospholipid composition, i.e., phosphatidic acid, diphosphatidylglycerol, phosphatidylglycerol, and aminoacyl derivatives of phosphatidylglycerol. Differences in growth phase did not seem to explain the differences between these results and those of Ibbott and Abrams (21), since comparison with previously published data of Vorbeck and Marinetti (49) concerning bacteria from the stationary phase pointed to quantitative but not qualitative changes. No effort appears to have been made to check possible influences of the different culture media, especially the presence or absence of glucose. Tentative identifications carried out by Houtsmuller and van Deenen (20) and Moore et al. (33) agreed more closely with the description of Vorbeck and Marinetti (50). Recently, the presence of a diglucosylglycerol residue in the phospholipid fraction was described (12). In our studies of the lipids extracted from *S. faecalis*, a more complex pattern of phospholipids was encountered. This paper describes their isolation and preliminary characterization.

[1] Present address: Serviço de Higiene e Medicina Social, Faculdada de Medicina, Porto, Portugal.

MATERIALS AND METHODS

Preparation of cells. *S. faecalis* ATCC 9790 was cultivated at 38 C, under continuous aeration, for 18 to 20 hr (stationary phase). The culture medium contained 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% dipotassium phosphate, and 1.0% glucose. The medium was adjusted to *p*H 7.0 before autoclaving. Glucose was added after sterilization. Labeling of the phospholipids was obtained by the addition of 200 μCi of ^{32}P-orthophosphate per 100 ml of medium. The cells were harvested by centrifugation, washed with distilled water, freeze-dried, and weighed.

Extraction of the lipids. Basically the procedure of Bligh and Dyer (3) was followed. Water, acidified to *p*H 4.5, was used to prevent possible decomposition of some phospholipids. Lyophilized cells were suspended in chloroform-methanol-water (1:2.1:1, v/v) and stirred for 30 min at room temperature. One volume of chloroform and one volume of water were added, and the mixture was stirred for another 30 min. After centrifugation, the upper phase was rejected and the lower phase, containing the lipid material, was collected. This procedure was repeated with the remaining interphase. The combined lipid extracts were dried in vacuo and weighed.

Isolation of the phospholipids. A first fractionation of the lipid material was achieved by chromatography on a silicic acid column. The column fractions corresponding to the apolar lipids and to the glycolipids

were dried, weighed, and discarded. The phospholipid fractions, containing more than one compound, were further purified by preparative thin-layer chromatography. Purified fractions, with identical chromatographic characteristics both on paper and on thin-layer chromatography, were assumed to represent the same compound and were combined. All extracts were concentrated by evaporation at 37 C under reduced pressure.

Column chromatography. A silicic acid column (Mallinckrodt Chemical Works, 100 mesh), was used for fractionation of the total lipid extract. Nonpolar lipids were eluted with chloroform and glycolipids with increasing concentrations of acetone in chloroform (49); remaining polar lipids (phospholipids) were eluted with a discontinuous gradient of methanol in chloroform.

Paper chromatography. Paper chromatography of lipid material was performed on silicic acid-impregnated paper, with diisobutylketone-acetic acid-water (8:5:1, v/v) as solvent system (solvent 1) (G. V. Marinetti, J. Erbland, and J. Kochen, Fed. Proc., p. 837, 1957). Paper chromatograms of total lipid extracts from ^{32}P-grown cells were scanned in front of an end-window Geiger-Muller tube, to calculate the relative amount of each compound. Descending paper chromatography on Whatman no. 1 paper was carried out with 1-propanol-concentrated ammonia-water (6:3:1, v/v; solvent 2), for water-soluble breakdown products of the phospholipids, and with *n*-butyl alcohol-acetic acid-water (5:2:3, v/v; solvent 3), for carbohydrates.

Chromatograms were stained with: (i) the tricomplex system (7), in which phospholipids with a positive charge next to the negative one of the phosphoryl group stained red, and acidic phospholipids stained green; (ii) a 0.5% solution of ninhydrin in 85% butanol, for free amino groups; (iii) an alkaline silver nitrate reagent (47) for carbohydrates; (iv) the periodate-Schiff reagent (1) for vicinal hydroxyl groups; and (v) the reagent of Hanes and Isherwood (14) for phosphate groups.

Thin-layer chromatography (TLC). The following solvent systems were used with silica gel G (Merck): chloroform-methanol-acetic acid-water (125:37:10:2, v/v; solvent 4) for phospholipids; ether-hexane (3:7, v/v; solvent 5) for apolar lipids (glycerides and fatty acids); and methyl-ethylketone-methanol-acetic acid (3:1:1, v/v; solvent 6) for carbohydrates.

Chromatograms were stained with: (i) iodine vapor (42) for lipid material; (ii) a 0.5% solution of ninhydrin in 85% butanol, to detect free amino groups; (iii) a 0.2% solution of naphthoresorcinol in 20% sulfuric acid, for carbohydrates; (iv) the periodate-Schiff reagent (1) for compounds having vicinal hydroxyl groups; and (v) a molybdate spray (10) for phosphate groups.

Gas-liquid chromatography (GLC). The trimethylsilyl derivatives of carbohydrates were chromatographed at 175 C, in a column of 15% polyethyleneglycol succinate on Chromosorb W (Applied Science Laboratories), mounted in a Pye Argon Chromatograph. The methyl esters of the fatty acids were chromatographed at 180 C in a column of 3% polyethyleneglycol succinate on Chromosorb O (Applied Science Laboratories) in an F and M gas chromatograph.

Ion-exchange chromatography. Ion-exchange chromatography was applied for the qualitative and quantitative analysis of amino acids by use of a Beckman Unichrom amino acid analyzer.

Paper electrophoresis. Paper electrophoresis, both of the intact lipids and their breakdown products, was carried out at 50 v/cm, on MN paper (Machery, Nagel Co., Düren, Germany) using, unless otherwise stated, a system of pyridine-acetic acid-water (1:10:89, v/v), *p*H 3.6.

Nonenzymatic hydrolysis. Deacylation of the phospholipids was performed by the method of Maruo and Benson (31). The phospholipids were dissolved in methanolic 0.1 N KOH, and incubated for 10 to 15 min at 37 C. The reaction was stopped by neutralizing with Dowex 50 (H^+) (J. T. Baker Chemicals), and the resin was subsequently removed by centrifugation. Lipid- and water-soluble products were separated with chloroform-2-methyl-1-propanol-water (4:2:3, v/v). The water-soluble material was compared with reference compounds by paper electrophoresis and paper chromatography (solvent 2). Phospholipids containing free amino groups were hydrolyzed in 0.01 M borate buffer, *p*H 9.0 (5, 20). A good emulsion was obtained by treatment of the phospholipid in the buffer, for 2 min, in a Branson Sonifier. After incubation for 1 hr at 37 C, lipid material was extracted with chloroform and tested by paper chromatography in solvent 1. The remaining water-soluble material was analyzed by ion-exchange chromatography to detect amino acids, with or without previous acid hydrolysis.

Alkaline hydrolysis of some deacylated fractions was performed in 5 ml of 0.1 N NaOH, at 100 C, for 1 hr. The resulting material was deionized with Dowex 50 (H^+). The resin was removed by centrifugation, and the liquid was evaporated under reduced pressure. The dry material was redissolved in a small amount of water and tested against reference compounds by paper chromatography with solvent 2.

Acid hydrolysis for the study of carbohydrates was carried out in 5 ml of 3 N HCl at 100 C for 90 min, in a sealed tube. The hydrolysates were passed through a column filled with a 1:1 mixture of Amberlite IR45 (OH^-) and Amberlite IRC50 (H^+) (L. Light and Co.), evaporated to dryness, and dissolved in pyridine. The pyridine solution was evaporated to dryness, and the extract was redissolved in water. Identification of the carbohydrate material was done by using reference compounds, by paper chromatography in solvent 3; by TLC in solvent 6; and by GLC after conversion into the trimethylsilyl derivatives (45).

Acid hydrolysis for the study of amino acids was performed in 6 N HCl, at 110 C for 24 hr, in a sealed tube. The hydrolysate was applied to a column of Dowex 50 W-X8 (H^+), washed with water, and eluted with 2 N NH_4OH (17). The ammonia solution was subsequently evaporated to dryness, and the resulting material, after repeated washings with distilled water, was dissolved in 0.05 N HCl and tested by ion-exchange chromatography.

Formation of formaldehyde upon oxidation with periodic acid (26) was carried out in ethanol with very concentrated solutions of the intact lipids in chloroform. For the quantitative estimation of periodate consumption, sodium periodate and the deacylated compounds were used instead, the disappearance of periodate being followed by spectrophotometry (11).

For fatty acid analysis, the phospholipids were hydrolyzed with 5% concentrated sulfuric acid in methanol at 70 C for 2 hr. The resulting methyl esters of the fatty acids were extracted with pentane and assayed by GLC.

Enzymatic hydrolysis. Hydrolysis with phospholipase A (EC 3.1.1.4) was carried out in a borate buffer, *p*H 7.0 (29) with 5.0×10^{-3} M calcium acetate, using a crude extract of *Crotalus adamanteus* (Sigma Chemical Co.). Hydrolysis with phospholipase C (EC 3.1.4.3), prepared from *Bacillus cereus* (8), and with phospholipase D (EC 3.1.4.4), obtained from Savoy cabbage, followed the description of Haverkate and van Deenen (15). Phospholipase D from peanuts, purified (stage 3) by the method of Heller et al. (18), was also used. The lipid-soluble breakdown products resulting from the action of phospholipases were tested by paper chromatography with solvent 1 and by TLC with solvent 5; the water-soluble material was tested by paper electrophoresis or paper chromatography with solvent 2 (or with both). Reference compounds were used in both instances. A 4% (w/v) solution of almond emulsin β-glucosidase (EC 3.2.1.21), from Sigma Chemical Co., in 0.05 M acetate buffer (*p*H 5.45), was incubated overnight at 37 C with the deacylated forms of carbohydrate containing phospholipids. Breakdown products were investigated by paper chromatography in solvents 2 and 3.

Assay procedures. Established procedures were used for the assay of phosphorus (13), glycerol (39), and fatty acid esters (43). Glucose was assayed both with the anthrone reagent (37) and with glucose oxidase (EC 1.1.3.4) from Merck (40). Spectrophotometry was used for the assay of nicotinamide adenine dinucleotide (NAD), at 340 nm, and sodium metaperiodate, at 227 nm, with a UNICAM SP 500 spectrophotometer. Lysine was also assayed microbiologically with *Leuconostoc mesenteroides* (44).

RESULTS

S. faecalis was harvested at the stationary phase of growth (*p*H 4.2). About 1 g of cells (dry weight) was obtained per liter of culture medium. Total lipids accounted for 1.2% of the cell dry weight. When the total lipid extract was applied on a silicic acid column, 25% of the total amount of lipid, consisting of apolar lipids, was eluted with chloroform. A fraction containing 26% of the total lipids was obtained by eluting the column with increasing concentrations of acetone in chloroform (49). No phosphorus could be detected in this fraction which corresponds probably to glycolipid. The remaining 49% of the lipids was eluted with increasing concentrations of methanol in chloroform. All compounds found in these eluates contained phosphorus.

Autoradiograms of lipid extracts from bacteria grown in the presence of ^{32}P, and chromatographed on silicic acid-impregnated paper, showed six major spots (Fig. 1). The corresponding phospholipids were designated as A, B, C, E, F, and G. Still another compound, occupying an intermediate position relative to C and E (compound D), was detected in trace amounts, but no attempt was made to elucidate its structure.

Comparison was made between chromatograms of the total lipid extracts and the column fractions obtained by elution with increasing amounts of methanol in chloroform. Compound A was eluted first, with a mixture containing 2% methanol. Compound B was mainly present in the fractions containing 6 to 8% methanol.

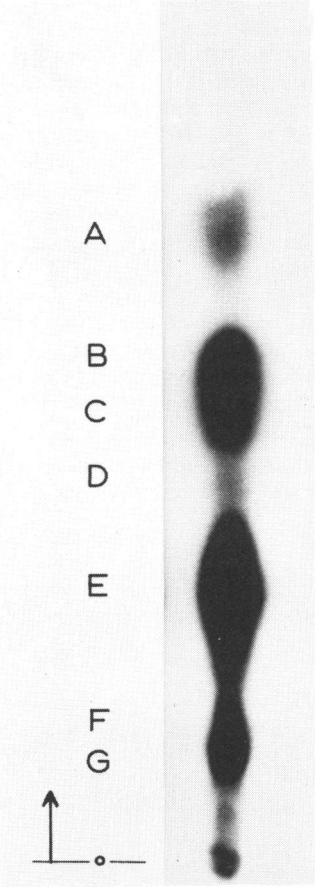

FIG. 1. *Autoradiogram of ^{32}P-labeled phospholipids of S. faecalis. The lipid extracts were separated on silica gel-impregnated paper in solvent system 1.*

Elution with 10 to 25% methanol gave mixtures of compounds C, E, and G. Compound F started to be eluted with 25% methanol.

Compound A. Compound A behaved, after deacylation, as a reference bis(glycerylphosphorylglycerol), both in its mobility and staining characteristics. Since the estimated phosphorus/glycerol ratio had a mean value of 1.0:1.5, it was tentatively identified as cardiolipin. Further support of this view was obtained by incomplete hydrolysis with phospholipase A. When the lipids resulting from phospholipase A degradation were compared by paper chromatography with the intact compound, three extra compounds were detected, one at the solvent front, and two others at about the same position as the lysoderivatives obtained by action of phospholipase A on ox heart cardiolipin.

Paper chromatograms in solvent 1 of the intact compound and of ox heart cardiolipin showed, for compound A, a somewhat lower R_F value (0.76 against 0.83). Since this could be the result of a higher content of unsaturated fatty acids in ox heart cardiolipin (Table 1), the two compounds were tested again in the same system after hydrogenation in the presence of platinum. The hydrogenation resulted in an appreciable reduction in the R_F value of both compounds, but especially in the case of ox heart cardiolipin for which the now-saturated form occupied a lower position on the paper as compared to the saturated form of compound A (Fig. 2). However, this result cannot be considered as definitively establishing a different structure between compound A and ox heart cardiolipin. It is possible that cyclopropane containing fatty acids present in compound A (about 20% of the total) may influence the chromatographic behavior in a way identical to unsaturated fatty acids.

TABLE 1. *Fatty acid composition of ox heart cardiolipin and of phospholipids from S. faecalis*

Fatty acid	Ox heart cardiolipin	Phospholipids from *S. faecalis*			
		A	B	E	F
	%	%	%	%	%
14:0		5.5	5.6	3.3	4.2
15:0			2.3		
16:0		30.0	38.1	38.4	36.3
16:1	4.6	7.4	6.8	5.7	13.2
16:2	1.2				
18:0			1.7		
18:1	5.8	36.7	32.7	38.5	43.8
18:2	81.4				
18:3	7.0				
19:0c[a]		20.2	12.6	14.1	2.3

[a] Tentative characterization as the C-19:0 cyclopropane fatty acid lactobacillic acid.

The ratio P/fatty acid esters (1.0:2.6) points, however, to a different diphosphatidylglycerol structure, with an extra fatty acid residue, i.e., a monoacyl derivative of diphosphatidylglycerol. On paper chromatography with solvent 1, this derivative could actually be expected to have a higher mobility, as found for compound A. The same suggestion was obtained after hydrolysis of compound A with phospholipase C, since chromatograms of the resulting chloroform-soluble material disclosed the presence of diglycerides and of two new polar compounds, as could be expected in the case of a pentaacyl structure (Fig. 3). Degradation, however, was too small, even in the presence of Zn^{2+} (A. C. Ottolenghi, Fed. Proc., p. 549, 1969), to allow the isolation and characterization of the breakdown products. Actually, the synthetic monoacyl derivative of diphosphatidylglycerol was reported (9) to be rather resistant to the action of phospholipase C.

Compound B. This phospholipid co-chromatographed with a reference phosphatidylglycerol from *Chlorella vulgaris* both on paper (solvent 1) and thin-layer (solvent 4). The P/glycerol ratio was found to be 1.0:2.2, and 1 mole of formaldehyde was produced per phosphate residue upon periodic acid oxidation. Furthermore, deacylation resulted in the formation of a water-soluble compound which appeared to be identical to glycerylphosphorylglycerol when tested both by paper chromatography (solvent 4) and paper electrophoresis. To clarify the stereochemical configuration of both glycerol moieties, experiments were performed as outlined by Haverkate and van Deenen (15). The products resulting from the action of phospholipase A (16) consisted of fatty acids and a compound similar to lysophosphatidylglycerol as shown by paper chromatography in solvent 1. Phospholipase A is able to remove the fatty acid esterified at the 2-position of the phosphoglyceride only in the case of 3-*sn*-phosphoglyceride (48).

Hydrolysis of compound B with phospholipase C resulted in the formation of diglyceride and a water-soluble compound which appeared to be identical to glycerophosphate both by paper chromatography (solvent 2) and paper electrophoresis. The glycerophosphate was not oxidized by a specific glycero-3-phosphate dehydrogenase (EC 1.1.1.8) as assessed by lack of conversion of NAD to reduced NAD (NADH). It can be concluded from these data that compound B is identical with 1,2-diacyl-*sn*-glycero-3-phosphoryl-1′-*sn*-glycerol.

Compound C. The R_F value of compound C on

paper chromatograms (solvent 1) appeared to be between those of phosphatidylglycerol and a reference lysylphosphatidylglycerol from *B. megaterium*. The red color obtained with the tricomplex system indicated the presence of a positive charge next to the negative phosphoryl group. A reaction with ninhydrin indicated the presence of free amino groups. After acid hydrolysis, the presence of lysine (80%) and alanine (20%) was detected. In some assays, small amounts of other amino acids were found, but their presence was not constant, so it is possible that they represent protein contamination of the phospholipid. Treatment of compound C with borate buffer followed by amino acid analysis of the water-soluble hydrolysis products also disclosed the presence of lysine (about 75%) and alanine (25%), whether or not the hydrolysate was subjected to acid hydrolysis prior to analysis. The lipid that could be isolated after borate hydrolysis co-chromatographed with phosphatidylglycerol. Deacylation of compound C by strong alkaline hydrolysis resulted in the formation of glycerylphosphorylglycerol. These results indicate that lysine and alanine residues are separately attached to phosphatidylglycerol. The results of the hydrolysis in borate buffer rule out the possibility that the amino acids are linked to the phosphatidylglycerol in the form of a peptide consisting of three lysine and one alanine residue, because it is unlikely that the mild alkaline conditions would break the peptide linkages. On the other hand, if one lysine and one alanine were present for each phosphatidylglycerol residue, each esterified to one of the two available hydroxyl groups of the terminal glycerol, we would expect a lysine/alanine ratio of 1:1 upon acid or borate hydrolysis. It is very likely, therefore, that compound C corresponds to a mixture of the lysyl- and the alanyl-derivatives of phosphatidylglycerol which have been described and chemically synthesized (4, 6, 20, 29).

Further experiments were carried out to check the possibility that one or both amino acids are not covalently bound to phosphatidylglycerol, but merely represent contaminating material. The intact lipid was subjected to paper electrophoresis with aqueous development systems of different *p*H values, as follows: (i) 0.75 N formic acid-1 N acetic acid (1:1, v/v), *p*H 2.25; (ii) pyridine-acetic acid–water (1:10:89, v/v), *p*H 3.6; (iii) 0.1 N acetate buffer, *p*H 6.0. The material remaining at the origin and the migrating fractions were eluted from the paper and hydrolyzed with HCl. Amino acid analysis of the hydrolysates showed that most of the lysine and alanine stayed at the origin during electrophoresis.

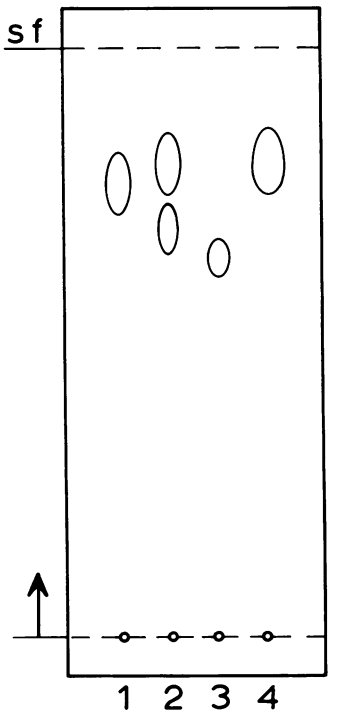

FIG. 2. *Chromatographic comparison of compound A (1), hydrogenated compound A (2), hydrogenated ox heart cardiolipin (3), and ox heart cardiolipin (4). Compounds were tested on silica gel-impregnated paper in solvent system 1.*

The presence of smaller amounts of the amino acids in the migrating fractions might be due to hydrolysis of the unstable amino acid-phospholipid linkage (5, 20). Paper chromatography (solvent 1) of synthetic alanyl phosphatidylglycerol showed that the R_F value of this compound is about the same as that of phosphatidylglycerol (4). Compound C was found to migrate slightly slower than phosphatidylglycerol, but it might be possible that interaction with a more polar compound (i.e., lysylphosphatidylglycerol) affects its migration. Furthermore, there is a difference in R_F value between the tentatively identified lysylphosphatidylglycerol in compound C and a reference 3′-lysylphosphatidylglycerol from *B. megaterium*. Molotkovsky and Bergelson (32) reported, however, that a synthetic 2′-isomer of lysylphosphatidylglycerol had a higher R_F value in the chromatographic system used than the 3′ isomer. A sample of synthetic 1,2 distearoyl-*sn*-glycerol-3-phosphoryl-1′-(2′-O-L-lysyl)glycerol provided by these investigators revealed a chromatographic behavior similar to compound C. Our results indicate, therefore, that compound C consists of a mixture of the

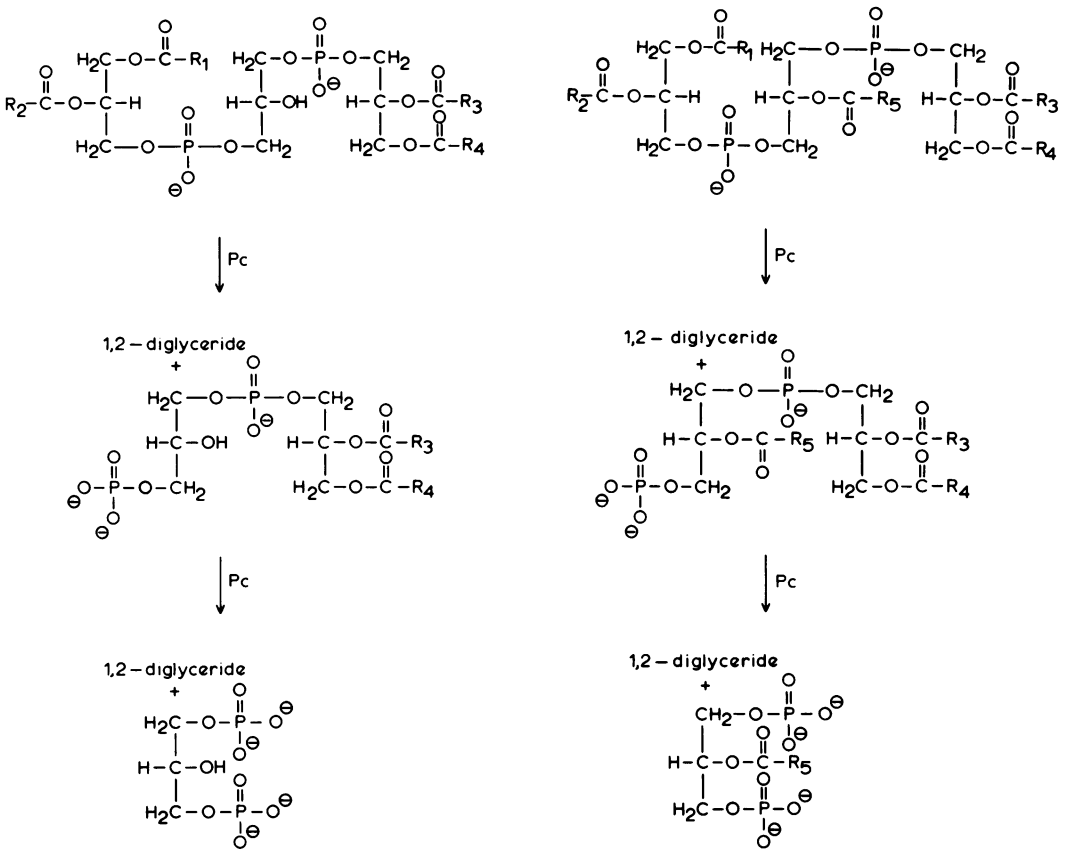

FIG. 3. *Degradation of diphosphatidylglycerol and its monoacyl derivative with phospholipase C.*

2′ isomer of lysylphosphatidylglycerol and alanylphosphatidylglycerol.

Compound E. The presence of a positive charge and of free amino groups was ascertained through the tricomplex and the ninhydrin stainings. Paper chromatography (solvent 1) showed that compound E migrated as a reference 3′-lysylphosphatidylglycerol from *B. megaterium*. The deacylated material, tested by paper electrophoresis and paper chromatography (solvent 2), disclosed the presence of glycerylphosphorylglycerol. On this basis, compound E was thought to represent a derivative of phosphatidylglycerol. After acid hydrolysis, amino acid analysis showed mainly the presence of lysine (up to 91%) and of arginine (up to 9%). The P/lysine ratio was found to be 1.0:0.9. That both amino acids were covalently bound to phosphatidylglycerol was indicated by the results of paper electrophoresis carried out with the intact lipid, at *p*H 3.6. Again, the lysine and arginine did not migrate from the origin, except for a very small amount of lysine originating probably from hydrolysis of the lipid. Hydrolysis of compound E in borate buffer resulted in the formation of a phospholipid which co-chromatographed with phosphatidylglycerol. The water-soluble hydrolysis products were investigated for their amino acid composition. Without previous acid hydrolysis, both lysine and arginine were the main amino acids which could be detected. The results show that both amino acids are separately linked to phosphatidylglycerol, suggesting compound E to be a mixture of the lysyl- and the arginyl-derivatives of phosphatidylglycerol.

Compound F. Compound F co-chromatographed with a reference diglucosylphosphatidylglycerol isolated by Ishizuka and Yamakawa (23) from *S. hemolyticus*. The water-soluble products formed upon deacylation of both compound F and the reference lipid appeared to be identical by paper electrophoresis and paper chromatography (solvent 2). Glucose was the only sugar detected after acid hydrolysis. The results of the glucose assays used (the anthrone method and the glucose oxidase reaction) indicate that all the glucose is in the D-form. The ratio P/fatty acid esters/glycerol/glucose

was found to be 1.0:2.2:2.0:2.1. These data agree with the diglucosyl (kojibiosyl) phosphatidylglycerol structure as described by Ishizuka and Yamakawa (23). Further support was obtained by other hydrolytic procedures. After hydrolysis of compound F with phospholipase D from cabbage, a lipid was isolated which chromatographed as a reference phosphatidic acid and yielded glycerophosphate upon deacylation.

Concerning the linkages glucose-glucose and glucose-glycerol, the C-1 of both glucose moieties seems to be blocked since the intact compound does not react with the alkaline silver nitrate reagent. Although no definitive studies are available as to the other carbon atom involved in the linkage between the two glucose moieties, quantitative measurements after periodate oxidation of the deacylated compound showed that 4.0 moles of sodium periodate were consumed per phosphate residue. This value agrees with the 1 → 2 linkage proposed by Ishizuka and Yamakawa (23), although the 1 → 4 linkage is also possible. No definitive evidence exists concerning which carbon atom of glycerol is linked to the disaccharide moiety, but since compound F is a derivative of phosphatidylglycerol (compound B), it is logical to assume that either C-2′ or C-3′ is involved. Results of alkaline hydrolysis seem to favor the involvement of C-3′. When deacylation of compound F was followed by an alkaline hydrolysis in 0.1 N NaOH, paper chromatography in solvent 2 showed the presence of glycerol phosphate and of a compound having an R_F value similar to that reported (23) for diglucosylglycerol. The purple color given by this compound with the periodate-Schiff staining, supports the presence in the glycerol moiety of two vicinal hydroxyl groups, thus suggesting that the disaccharide moiety is linked to a terminal carbon atom of the glycerol residue (C-3′). As to the type of glycosidic bond, the α-type seems to be involved, since β-glucosidase failed to increase liberation of glucose from the deacylated compound, as compared to a control in which no enzyme had been added. In both cases only small amounts of free glucose were detected.

Compound G. Present in small amounts was another ninhydrin-positive compound in which amino acid analysis after acid hydrolysis showed predominantly the presence of lysine (86%). A great variety of amino acids made up the remaining percentage, but the high polarity of this compound makes it likely that some contaminating material remains even after purification on TLC. Paper electrophoresis and paper chromatography in solvent 2 of the deacylated compound showed the presence of glycerylphosphorylglycerol and of some ninhydrin-positive material migrating as a reference lysine. After hydrolysis in borate buffer, a compound corresponding chromatographically to phosphatidylglycerol was detected. Amino acid analysis of the water-soluble fraction carried out without previous acid hydrolysis disclosed mainly the presence of lysine (85%). Whereas these results point to a lysyl derivative of phosphatidylglycerol, the ratio P/lysine (1.0:2.1) suggests that two lysine residues are attached to each phosphatidylglycerol residue. Since it is unlikely that a peptide bond between two lysine residues is broken during the borate treatment, the remaining hypothesis seems to be that the two lysine moieties are esterified adjacent to the hydroxyl groups of the terminal glycerol. Compound G was subjected to hydrolysis with a partially purified preparation of phospholipase D from peanuts (18). The resulting lipid was identified as phosphatidic acid; the water-soluble fraction contained lysine and glycerol in a ratio 1.0:0.6. Paper electrophoresis of this water-soluble fraction revealed one major spot moving to the negative pole. The compound reacted with ninhydrin but not with the periodate-Schiff reagent. This supports the view that both lysine residues are directly linked to glycerol.

Fatty acid composition. The fatty acids of some of the isolated compounds were tentatively identified on the basis of their relative retention times on GLC (Table 1). The general pattern agrees with other descriptions of the fatty acids of *Lactobacillaceae* (30, 46), which reported the dominance of the 16:0, 18:1, and 19:0 cyclopropane-containing fatty acids. Although we have not yet proven the presence of the 19:0 cyclopropane fatty acid, its identity seems likely on the basis of the relative retention time on GLC. The low amount of this fatty acid present in compound F, as compared to the other compounds, is remarkable. The cyclopropane-containing fatty acids were reported to be synthesized directly on the phospholipid molecule (51). The possibility that the low value of this fatty acid in compound F is related to a lower affinity of its synthetase to diglucosylphosphatidylglycerol is perhaps worth attention.

DISCUSSION

The phospholipid composition of *S. faecalis* differs, in our experimental conditions, from other reports concerning this organism. That some of these reports deal with phospholipids of the membrane fraction, whereas our results are obtained with extracts of the whole organism, does not explain the observed differences, because it was shown (27) that no more than 4% of the total lipid phosphorus may be present in the soluble

cell fraction and the cell wall. Ibbott and Abrams (21) detected in the same strain of *S. faecalis* only two polar lipids, one accounting for 54% of the total lipids and corresponding probably to diphosphatidylglycerol; the other one (23%) was shown to contain carbohydrates. Although no identification of this last compound was made, it is possible that it corresponds to our compound F, in spite of the high ratio of P/fatty acid esters (1:6) reported. The high amount of diphosphatidylglycerol could well be related to our observation (*unpublished results*) that omission of glucose from the culture medium results in a remarkable increase of a compound corresponding in its chromatographic position to compound A. The results of Vorbeck and Marinetti (50), which described phosphatidic acid, diphosphatidylglycerol, phosphatidylglycerol, and aminoacylphosphatidylglycerol, are closer to our own, although the culture medium and the phase of growth (exponential), factors which are known to affect the phospholipid pattern of bacteria (19, 24, 34, 35, 38), were quite different. We could not, however, identify phosphatidic acid in our extracts.

A pentaacyl structure for diphosphatidylglycerol was suggested (21) but has not been proved. In our case, results of chromatography, P/fatty acid esters ratio, and hydrolysis with phospholipase C are also suggestive of such a structure, but possible effects of a different fatty acid composition and of contamination with small amounts of glycerides cannot be completely ruled out. Further experiments will be carried out to establish the structure of this phospholipid.

The stereochemical configuration we found for phosphatidylglycerol supports the work of Benson and Miyano (Biochem. J. **81**:31P, 1961) who proposed this configuration for the natural compound, and is in agreement with the biosynthetic pathway of this phospholipid (25, 26). Although no studies were carried out on the stereochemical configuration of the phosphatidylglycerol moiety of the newly found derivatives of the phosphatidylglycerol, it seems likely that it corresponds to that of compound B.

The reported ninhydrin-positive compounds all seem to represent derivatives of phosphatidylglycerol. As for compound C, our results suggest that we are dealing with a mixture of alanylphosphatidylglycerol and of the 2′-isomer of lysylphosphatidylglycerol. This last compound was not detected before in nature, but has already been synthesized (32) and was shown to have a higher R_F value on diisobutylketone-acetic acid-water, than the 3′-isomer. The possibility that it represents a degradation product of compound G cannot be ruled out. Compound E seems also to represent a mixture of aminoacyl derivatives of phosphatidylglycerol, the lysyl-ester (in this case the 3′-isomer) and the arginyl-ester. The 3′-isomer of lysylphosphatidylglycerol has been known for some time (20, 29, 36) and was chemically synthesized (6). The work of Lennarz et al. (7th Int. Congr. Biochem., Tokyo, Abstr. IV, pg. 737, 1967) already suggested that the arginyl derivative may be present in this organism. Further experiments are necessary to extend the evidence that this phospholipid occurs in *S. faecalis*. It is well known that hexose-containing phospholipids exist as chemical entities in natural compounds (for a review see reference 36), although Lefevre et al. (28) recently called attention to the weakly associated complexes of monosaccharides with phospholipids. The glucose-containing compound F seems to correspond to the diglucosylphosphatidylglycerol recently reported in *S. hemolyticus* (21) and possibly also in *S. faecalis* and *S. lactis* (12). Our studies on compound G point to a 2′,3′-dilysylphosphatidylglycerol structure which has not been described before. A chemical synthesis of this phosphoglyceride is in progress.

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