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Intracellular Distribution and Characterization of the Lipids of *Streptococcus faecalis* (ATCC 9790)*

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ABSTRACT: The lipids of exponential phase *Streptococcus faecalis* cells have been studied with respect to their intracellular distribution and nature. Subcellular fractions were prepared by enzymatic disintegration with muramidase followed by differential centrifugation of the released cell constituents. The membrane fraction contained 94% of the total cell lipid with the remainder in the protoplasm fraction. Lipids were not detected in the cell wall fraction. No differences were observed in the chemical and chromatographic properties of lipids extracted from either the membrane or protoplasm. The major components obtained by silicic acid column chromatography were identified as phosphatidyl glycerol, amino acid esters (lysine, glycine, and alanine) of phosphatidyl glycerol, monoglucosyl diglyceride, and a glycosyl diglyceride containing both glucose and galactose. Phosphatidic acid and diphosphatidyl glycerol (cardiolipin) were minor components. These lipids were identified by determination of chromatographic behavior, staining characteristics with specific reagents, ester/sugar ratios, and by the analysis of the water-soluble products obtained by partial hydrolysis.

Recent studies in this laboratory on the lipids of *Streptococcus faecalis* (ATCC 9790), *Lactobacillus plantarum* (Vorbeck and Marinetti, 1964, 1965), and other lactic acid bacteria[1] revealed considerable amounts of carbohydrate-containing lipids to be present. The chromatographic and staining properties of these lipids were similar to those of the galactolipids (monogalactosyl and digalactosyl diglycerides) observed in photosynthetic tissues (Benson *et al.*, 1958; Zill and Harmon, 1962), wheat flour lipids (Carter *et al.*, 1956), leaf lipids (Kates and Eberhardt, 1957; Kates, 1960), and grasses (Weenink, 1961). Kolb *et al.* (1963) found the phosphatides of *S. faecalis* localized in the cell membrane; however, little is known about either the nature of the individual phosphatides or the distribution and nature of glycosyl diglycerides within the bacterial cell.

In the present study with *S. faecalis*, both phosphatides and nonphosphatides have been investigated. Data are presented on the intracellular distribution and characterization of the major lipid components. Fractionation of the cell constituents preceded extraction and characterization of the lipids. Subcellular fractions were prepared by enzymatic disintegration with muramidase (E. C. 3.2.1.17, formerly called lysozyme) and subsequent fractionation of the released cell constituents by differential centrifugation. The lipid components were identified by chromatographic behavior, staining properties with specific reagents, radioisotopic data, and by examination of the water-soluble products formed on hydrolysis.

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[1] N. L. Vorbeck and G. V. Marinetti, unpublished observations.

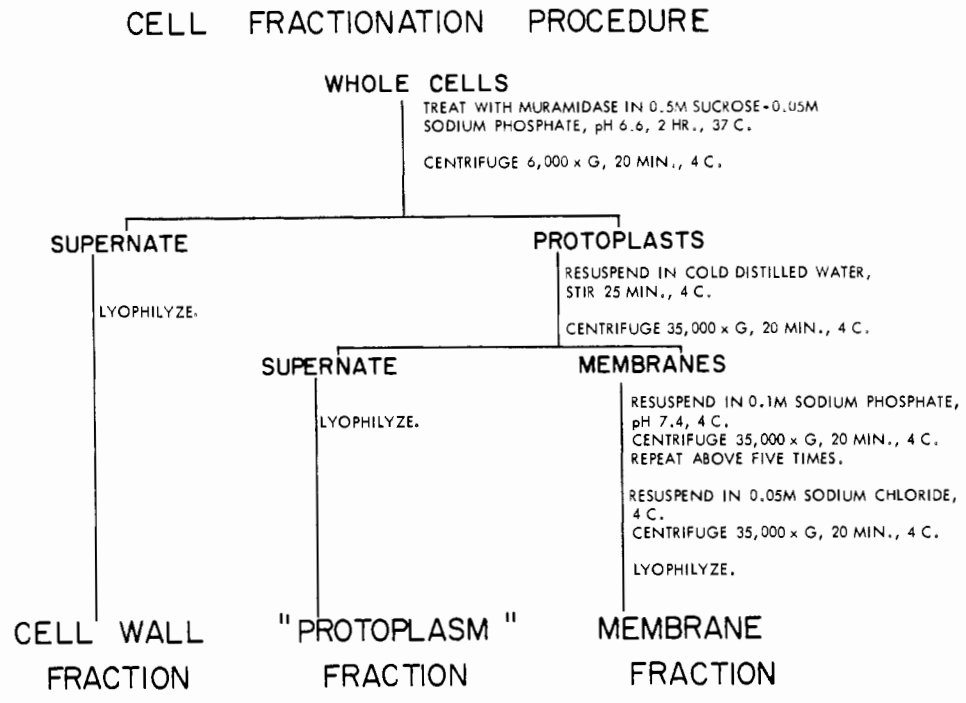


FIGURE 1: Procedure used for preparation of subcellular fractions.

Experimental

Preparation of Subcellular Fractions. S. faecalis (ATCC 9790) was grown at 37° in a lipid-free semisynthetic medium (Vorbeck and Marinetti 1965). Cells from the exponential phase of growth were harvested and washed three times with cold distilled water. The procedure used to prepare the subcellular fractions is outlined in the flow diagram in Figure 1. Washed cells (10 g), suspended in 600 ml of 0.5 M sucrose containing 0.05 M sodium phosphate buffer (*p*H 6.6), were treated with 75 mg of crystalline muramidase (Worthington Biochemical Corp., Freehold, N.J.). The suspension was placed in a water bath at 37° for 2 hours. During the incubation period, the cells were converted to protoplasts which were osmotically stabilized by the high sucrose concentration. All subsequent operations were performed in the cold (0–5°). The suspension was centrifuged at 6000 × *g* for 20 minutes and the supernatant fluid, containing the bulk of the cell wall material, was removed and lyophilized. The pellet, consisting of the protoplasts, was resuspended in 500 ml of cold distilled water and stirred vigorously for 25 minutes. Lysis of the protoplasts occurred immediately, resulting in the formation of membrane "ghosts" and in the release of soluble "protoplasm." The lysed protoplast suspension was centrifuged at 35,000 × *g* for 20 minutes and the supernatant containing the soluble "protoplasm" was removed. The pellet, consisting of the membrane fraction and entrapped protoplasmic material, was washed by resuspension in 0.1 M sodium phosphate buffer (*p*H 7.4) and the mixture was centrifuged at 35,000 × *g* for 20 minutes. The supernatant fluid was decanted and combined with the soluble "protoplasm." The combined supernatants were lyophilized and constituted the soluble "protoplasm" fraction. The pellet containing the membrane fraction was washed with the 0.1 M phosphate buffer a total of six times. Following a final washing with 0.05 M sodium chloride, the membrane preparation was lyophilized.

Lipid Extraction and Silicic Acid Column Chromatography. Lipids from the various subcellular fractions were extracted with chloroform-methanol (2:1, v/v) as previously described (Vorbeck and Marinetti, 1965). The lipids were dissolved in chloroform to a known volume and aliquots were taken for dry weight determinations. Unisil silicic acid (Clarkson Chemical Co., Williamsport, Pa.) was used for the fractionation of the lipid components. Nonpolar lipids were eluted as a single fraction with chloroform. Polar lipids were fractionated by discontinuous elution with increasing concentrations of acetone in chloroform, followed by increasing concentrations of methanol in chloroform (Vorbeck and Marinetti, 1965). Five-ml fractions were collected by means of an automatic fraction collector. Details of the solvents used are given under Results.

Analytical Procedures. Phosphorus and carbohydrate analyses of the column fractions were performed as described elsewhere (Vorbeck and Marinetti, 1965). Ester analyses were done by the method of Rapport and Alonzo (1955). Nitrogen was determined by Nesslerization following digestion with perchloric acid (Marinetti *et al.*, 1957). The peak fractions were chromatographed on silicic acid–impregnated paper (Marinetti, 1962). Diisobutyl ketone–acetic acid–water

(40:20:3, v/v/v) and diisobutyl ketone–pyridine–water (54:40:6, v/v/v) were used for the separation of polar lipids, and heptane–diisobutyl ketone–acetic acid (96:6:0.5, v/v/v) was used for the separation of the nonpolar lipids. The chromatograms were stained with Rhodamine 6G for detection of all lipid components, with ninhydrin for amino lipids, and with phosphomolybdic acid–stannous chloride for choline-containing lipids (Marinetti, 1962). A modified periodate-Schiff reaction was used to detect lipids containing vicinal hydroxyls (Vorbeck and Marinetti, 1965).

Deacylation of the column fractions was performed by the Maruo and Benson (1959) modification of the Dawson procedure. Phosphatide fractions were hydrolyzed with 1 N hydrochloric acid for 10 minutes at 100° while the glycosyl diglycerides and nonreducing glycosides were treated with 3 N hydrochloric acid for 90 minutes at 100°.

The water-soluble components obtained by deacylation and/or acid hydrolysis of the column fractions were separated by paper chromatography. Identification was based on cochromatography with known authentic compounds, specific staining procedures, or comparison of the R_F values obtained with those reported in the literature for known compounds.

Water-soluble phosphate esters were separated by two-dimensional chromatography on Whatman No. 1 paper using phenol-water (100:38, w/v) in the first direction and butanol–propionic acid–water (142:71:100, v/v/v) in the second direction (Benson and Strickland, 1960). The spots were located by modification of the Wade and Morgan procedure (1953). Thoroughly dried chromatograms were immersed in a solution of ferric chloride in acetone.[2] The chromatograms were dried in a hood for several minutes and then immersed in a 1.25% solution of sulfosalicylic acid in acetone. ^{32}P-labeled esters were detected by autoradiography using Kodak Blue Brand X-ray film.

The nonreducing glycosides obtained by deacylation of the glycosyl diglycerides were separated by ascending chromatography in butanol-pyridine-water (3:2:1.5, v/v/v, Carter *et al.*, 1956). The alkaline silver nitrate reagent of Trevelyan *et al.* (1950) was used for the location of the spots.

Paper chromatography of the sugars was carried out by the ascending technique using butanol-pyridine-water (45:25:40, v/v/v) and ethyl acetate–pyridine–water (30:21:9, v/v/v). Multiple development (Thoma, 1963) was used to obtain complete resolution of glucose and galactose. The periodate-Schiff procedure of Buchanan *et al.* (1950) was used for detection of vicinal hydroxyls, and the aniline-hydrogen phthalate reagent (Partridge, 1949) was used for detection of reducing sugars.

Amino acids were separated by two-dimensional chromatography on Whatman No. 1 paper. Methanol-water-pyridine (80:20:4, v/v/v) was used in the first direction and butanol-acetone-water-dicyclohexylamine (40:40:20:8, v/v/v/v) was used in the second direction (Richards, 1964). The spots were made visible by treatment with ninhydrin.[3]

Enzymatic procedures for the qualitative and quantitative determination of glucose and galactose were carried out employing the Glucostat and Galactostat reagents of Worthington Biochemical Corp., Freehold, N.J.

Results

Distribution of Lipids in Subcellular Fractions. The lipid content of the subcellular fractions and the relative contribution of each fraction to total cell weight and total cell lipid is given in Table I. The data show a localization of approximately 94% of the total cell lipids in the membrane. No lipid was found in the cell wall. Lipid constitutes 30.4% of the membrane and contains 2.5% phosphorus, 1.5% nitrogen, and 2.4% carbohydrate. The protoplasmic lipids were similar to the membrane lipids in chemical composition and paper and column chromatographic properties.

TABLE I: Analysis of Cell Fractions of *Streptococcus faecalis*.[a]

Cell Fraction	Per Cent of Total Cell (dry wt)	Per Cent of Total Lipids (dry wt)	Per Cent Lipid in Each Fraction[b]
Cell wall	31.2	0	0
Membrane	8.9	94.1[c]	30.4
"Protoplasm"	57.4	5.9	0.3

[a] Average of two determinations. [b] Whole cells contain 2.9% lipid. [c] Chemical composition of membrane lipid: 2.5% P; 1.5% N; 2.4% total carbohydrate (estimated from anthrone reaction, galactose standard).

Paper Chromatographic Separation of Membrane Lipids. The paper chromatographic separation of the membrane lipids obtained from cells grown in the presence of [^{32}P]orthophosphate is illustrated in Figure 2. In the first column, autoradiography was used to locate the phosphorus-containing lipids. The major phospha ides, identified by subsequent procedures, were phosphatidyl aminoacyl glycerol (spot 1) and phosphatidyl glycerol (spot 3). The minor components were diphosphatidyl glycerol, which was not completely

[2] The ferric chloride reagent was prepared by dissolving 1.5 g $FeCl_3 \cdot 6\ H_2O$ in 970 ml acetone and 30 ml 0.3 N hydrochloric acid.

[3] The ninhydrin reagent consisted of 0.25% ninhydrin and 7% glacial acetic acid in acetone. The chromatograms were heated for 3 minutes at 75°.

TABLE II: Characterization of the Major Glycosyl Diglycerides of *Streptococcus faecalis*.

Procedure	Fraction A[a]	Fraction B[a]
Paper chromatography (R_F) (DAW system)[b]	0.70	0.37
Rhodamine 6G test	Yellow	Yellow
Ninhydrin test	−	−
Periodate-Schiff test	+	+
Deacylation[c] (mild KOH)	G-GLU,[d] FA (nonreducing)	G-(GAL,GLU),[d] FA (nonreducing)
HCl hydrolysis (3 N, 90 min, 100°)	G,[d] GLU,[d] FA	G,[d] GAL,[d] GLU[d] FA
Sugars released by acid hydrolysis	GLU[d,e]	GAL,[d,e] GLU[d,e]
Ester/sugar molar ratio	1.98	1.01

[a] Abbreviations used: G, glycerol; GLU, glucose; GAL, galactose; FA, fatty acid. [b] DAW = diisobutyl ketone–acetic acid–water, 40:20:3. [c] Acid hydrolysis of nonreducing glycoside from A released glycerol and glucose; acid hydrolysis of nonreducing glycoside from B released glycerol, glucose, and galactose. [d] Identified by chromatographic procedures. [e] Identified by enzymatic procedures.

resolved from phosphatidyl glycerol, and is seen as a "cap" on the phosphatidyl glycerol spot (spot 3), and phosphatidic acid (spot 5). The next column shows a tracing of the chromatogram stained with Rhodamine 6G for detection of all lipid components. The yellow-staining spot (spot 6) at the solvent front is nonpolar lipid. In addition to the phosphorus-containing lipids mentioned above, the carbohydrate-containing lipids are represented by the two yellow-staining spots (spots 2 and 4). The result of the ninhydrin test for free amino groups is shown in the third column. Phosphatidyl aminoacyl glycerol was the only component which gave a positive reaction. Lipids containing vicinal hydroxyls, which are detected by the periodate-Schiff test, are shown in the last column. The carbohydrate-containing lipids and phosphatidyl glycerol gave a positive test. The "cap" of diphosphatidyl glycerol did not give a positive periodate-Schiff test. No choline-positive material was observed in any component.

Silicic Acid Column Separation of Membrane Lipids. Lipids extracted from the membrane fraction were separated on Unisil silicic acid using discontinuous elution. The nonpolar lipids, eluted as a single fraction with chloroform alone, accounted for less than 5% of the total lipids. Chromatography of this fraction on silicic acid–impregnated paper using heptane–diisobutyl ketone–acetic acid (96:6:0.5) as the solvent indicated the presence of free fatty acids and some triglycerides.

The elution pattern of the polar lipids from the membrane fraction is shown in Figure 3. Fractions A and B contained glycosyl diglycerides while the phosphatides were found in fractions C, D, E, and F. The last fraction, F, is due to the marked increase in polarity of the eluting solvent. The peak fractions were chromatographed on silicic acid–impregnated paper and were subjected to the staining procedures outlined for Figure 2. The spots shown in Figure 3 represent the components of the single peak fractions. On the basis of their mobility and staining characteristics, the glycosyl

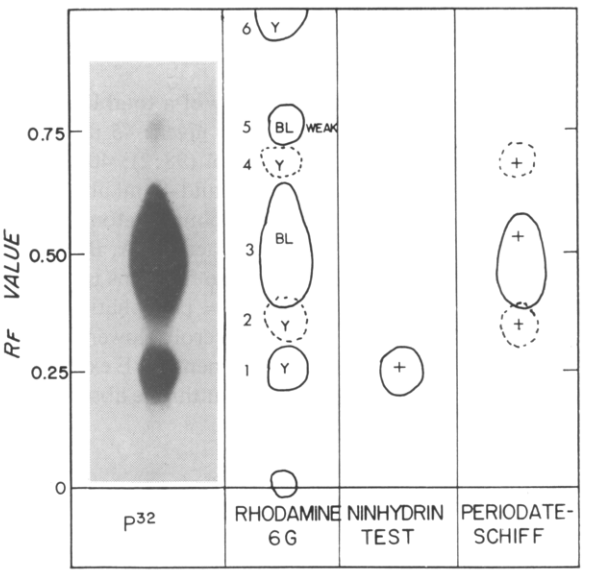


FIGURE 2: Separation of *S. faecalis* lipids on silicic acid–impregnated paper using diisobutyl ketone–acetic acid–water (40:20:3) as the solvent. The lipids were extracted from a membrane fraction prepared from cells grown in the presence of [^{32}P]orthophosphate. The lipid components are as follows: 1 = phosphatidyl aminoacyl glycerol, 2 = glucosyl galactosyl diglyceride, 3 = phosphatidyl glycerol, 4 = glucosyl diglyceride, 5 = phosphatidic acid, 6 = neutral lipids (triglycerides and fatty acids). The cap on spot 3 is believed to be diphosphatidyl glycerol (cardiolipin).

diglycerides in peak fractions A and B correspond to spots 4 and 2, respectively (Figure 2). A weakly staining blue component with an R_F value similar to that of spot 5 (Figure 2) was observed on chromatography of peak fraction C. The Rhodamine 6G and periodate-

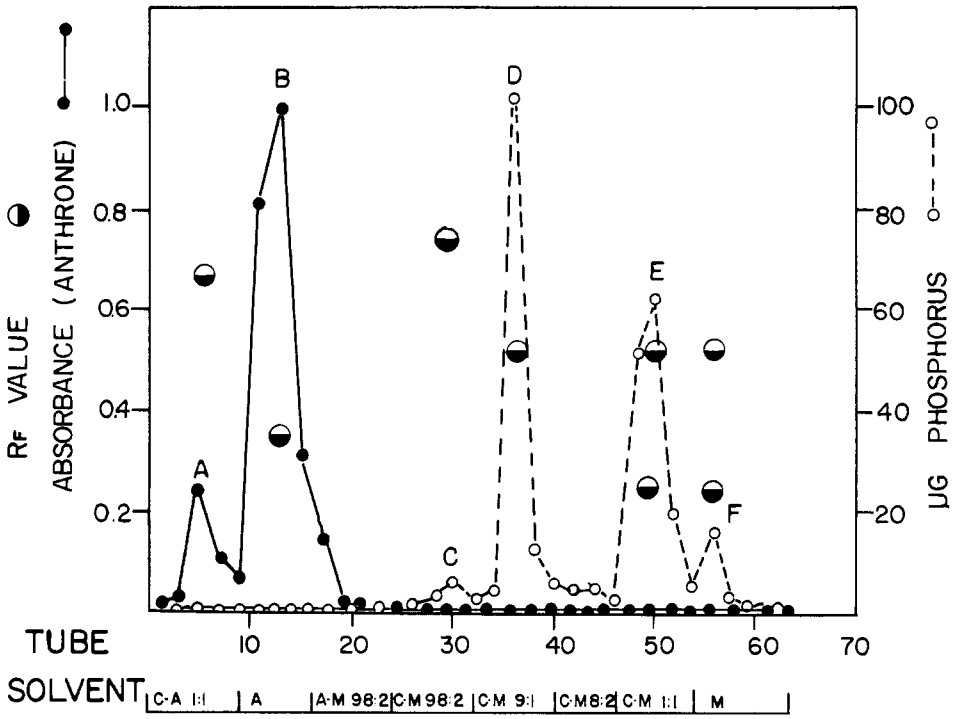


FIGURE 3: Column chromatography of a total lipid extract of the membrane fraction of *S. faecalis*. Elution was with the following solvents in the order given: 45 ml chloroform-acetone (1:1); 35 ml acetone, 40 ml acetone-methanol (98:2); 40 ml chloroform-methanol (98:2); 40 ml chloroform-methanol (9:1); 30 ml chloroform-methanol (8:2); 40 ml chloroform-methanol (1:1); and 40 ml absolute methanol. The peak fractions were chromatographed on silicic acid–impregnated paper using diisobutyl ketone–acetic acid–water (DAW) (40:20:3) for development. The staining characteristics and other tests of fractions A, B, D, E are given in Tables II and III. Fraction F stained the same as Fraction E. The identification of the fractions is as follows: A = glucosyl diglyceride, B = glucosylgalactosyl diglyceride, C = phosphatidic acid, D = phosphatidyl glycerol (also has very small amount of diphosphatidyl glycerol, E = phosphatidyl aminoacyl glycerol (slower component) plus a small amount of phosphatidyl glycerol (faster component), and F = same components as E except that both components occur in about equal amount. The ordinate refers to both the R_f value and the anthrone absorbance.

Schiff staining characteristics of peak fraction D were similar to those observed for spot 3 of Figure 2. The major slow-moving component in peak fraction E was ninhydrin positive and represents spot 1, whereas the properties of the minor faster-moving spot were similar to those of peak fraction D. The characteristics of peak fraction F were the same as those of peak fraction E. For further studies, the tubes were combined as follows: A, 3–8; B, 9–20; C, 27–32; D, 33–41; E, 45–53; F, 54–58.

Identification of Lipid Components. GLYCOSYL DIGLYCERIDES. The two carbohydrate-containing lipids, fractions A and B, obtained from the column fractionation were characterized further as glycosyl diglycerides by examination of the water-soluble products obtained by hydrolytic procedures. These results together with the staining properties of the intact lipids are summarized in Table II. The absence of free amino groups in both components is indicated by the negative ninhydrin reaction. Both A and B gave a positive periodate-Schiff test indicating vicinal hydroxyls. The water-soluble components formed by deacylation of A and B with mild KOH were nonreducing. The R_F values (butanol-pyridine-water, 3:2:1.5) were 0.44 and 0.25, respectively. Acid hydrolysis of the nonreducing glycoside obtained by deacylation of A released glycerol and glucose, whereas glycerol, glucose, and galactose were released by acid hydrolysis of the nonreducing glycoside obtained by deacylation of B. These same water-soluble components also were obtained when fractions A and B were hydrolyzed with acid. The glucose and galactose released by acid hydrolysis of fraction B were present in stoichiometric amounts as determined by quantitative enzymatic procedures. The ester/sugar ratio was 2:1 for fraction A and 1:1 for fraction B.

PHOSPHATIDES. The major phosphatide components obtained from the silicic acid fractionation, phosphatidyl glycerol (fraction D) and phosphatidyl aminoacyl glycerol (fraction E), also were characterized further by examination of the water-soluble moieties obtained by mild alkaline and acid hydrolysis. These results, in conjunction with R_F values and the staining behavior of the intact phosphatides with specific reagents, are sum-

TABLE III: Characterization of the Major Phosphatides of *Streptococcus faecalis*.[a]

Procedure	Fraction D	Fraction E
Paper chromatography (R_F) (DAW system)[b]	0.55	0.27
Rhodamine 6G test	Blue	Blue
Periodate-Schiff test	+	−
Ninhydrin test	−	+
Amino acids	None	Lysine[c,d] (alanine,[c,d] glycine[c,d])
Deacylation (mild KOH)	GPG,[c,d] FA	GPG,[c,d] AA,[c,d] FA
HCl hydrolysis (1 N, 10 min, 100°)	GP,[c,d] glycerol,[c,d] FA	GP,[c,d] glycerol,[c,d] AA,[c,d] FA

[a] Minor amounts of phosphatidic acid and diphosphatidyl glycerol (cardiolipin) also were detected. [b] DAW = diisobutyl ketone–acetic acid–water, 40:20:3. [c] Identified by chromatographic procedures. [d] Abbreviations used: GPG, glycerylphosphoryl glycerol; FA, fatty acid; AA, amino acid; and GP, glycerol phosphate.

marized in Table III. Fraction E (Figure 3) was rechromatographed on a Unisil silicic acid column to remove the faster-moving component. Fraction D was periodate-Schiff positive and ninhydrin negative; however, fraction E gave a negative periodate-Schiff test and a strong ninhydrin reaction. As illustrated by the autoradiogram shown in Figure 4, mild alkaline hydrolysis of fraction D released mainly glycerylphosphoryl glycerol and some 1,3-diglycerylphosphoryl glycerol. When fraction E was deacylated with mild KOH, glycerylphosphoryl glycerol was the only phosphate diester released. Both fractions D and E yielded glycerol phosphate and glycerol on acid hydrolysis. Since fraction E gave a positive ninhydrin test, the hydrolysates were examined for the presence of amino acids. Lysine and some alanine and glycine were detected by paper chromatography in both the alkaline and acid hydrolysates of this fraction.

A small amount of 1,3-diglycerylphosphoryl glycerol was found in the alkaline hydrolysate of fraction D (Figure 4) indicating the presence of diphosphatidyl glycerol. Although fraction C was detected in only trace amounts by the column fractionation, the autoradiogram (Figure 2) of the total lipids showed a fast-moving phosphatide with an R_F value similar to that reported in the literature for phosphatidic acid. Chromatography of the water-soluble phosphate esters released by mild KOH deacylation of the total ^{32}P-labeled membrane lipids (Figure 5) showed the presence of a very small amount of glycerol phosphate which would be expected to arise from phosphatidic acid.

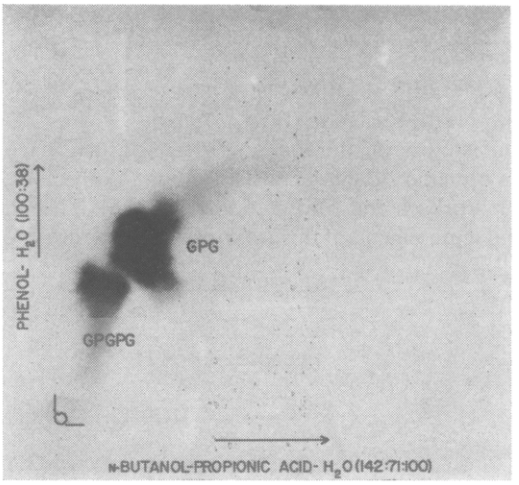


FIGURE 4: Autoradiogram of the methanolic KOH hydrolysate of fraction D obtained from the silicic acid column chromatography of *S. faecalis* membrane lipids. Fraction D was eluted from the column with chloroform-methanol (9:1). Abbreviations used: GPG, glycerylphosphoryl glycerol; and GPGPG, 1,3-diglycerylphosphoryl glycerol.

Discussion

Lipid analyses of subcellular fractions of Gram-positive and Gram-negative bacteria have revealed marked differences in both content and distribution. In general, Gram-positive bacteria contain less lipid than Gram-negative bacteria. Little or no lipid is found in the cell wall of Gram-positive organisms (Salton, 1960) and the phosphatides appear to be concentrated in the cell membrane (Weibull, 1957; Gilby *et al.*, 1958; MacFarlane, 1961; Yudkin, 1962; Kolb *et al.*, 1963). In contrast, Gram-negative bacteria contain 11–22% lipid in the cell wall (Salton, 1960). These observations have been the subject of numerous investigations concerning the mechanism of the Gram stain (Bergh *et al.*, 1964). Hofmann *et al.* (1952, 1955) have studied extensively the fatty acids of a variety of Gram-positive bacteria. However, with the exception of the aerobic sporeformers (Kates *et al.*, 1962; Houtsmuller and Van Deenen, 1963; Matches *et al.*, 1964)

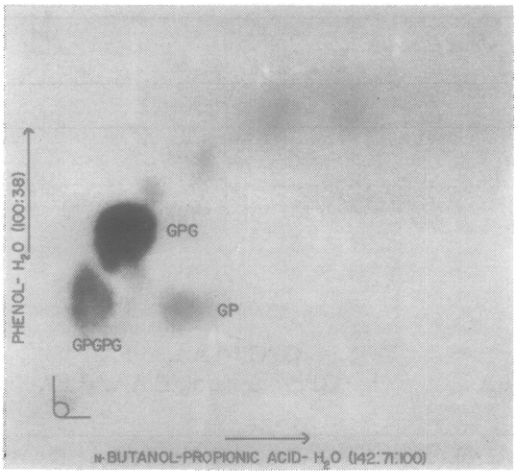


FIGURE 5: Autoradiogram of the methanolic KOH hydrolysate of total phosphatides of *S. faecalis* membranes. Abbreviations used: GP, glycerol phosphate; GPG, glycerylphosphoryl glycerol; GPGPG, 1,3-diglycerylphosphoryl glycerol.

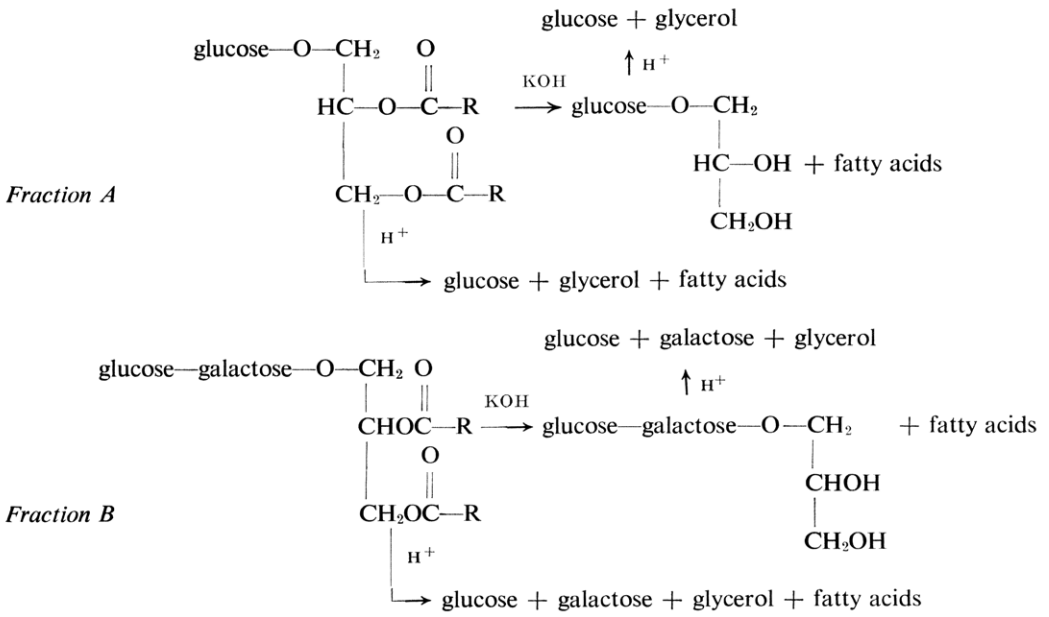
relatively little is known about the complex lipids of Gram-positive bacteria (Ikawa, 1963).

Our studies on the lipids of Gram-positive bacteria have centered on the lactic acid group (Vorbeck *et al.*, 1963; Vorbeck and Marinetti, 1964, 1965). The nutritional requirements of this group of organisms are known in great detail (Snell, 1948, 1952). Several species grow well in the absence of fatty acids, provided biotin is supplied, whereas othes require fatty acid (as oleic) even in the presence of biotin (Williams *et al.*, 1947; Kitay and Snell, 1950). Of the group, *S. faecalis* is sensitive to muramidase (Abrams, 1958) which provides a convenient method for preparing subcellular fractions. The present method differs from that used by Abrams *et al.* (1960, 1964) in that different buffers and different growth phase were used and the membranes were washed with phosphate buffer and NaCl and not with $MgSO_4$.

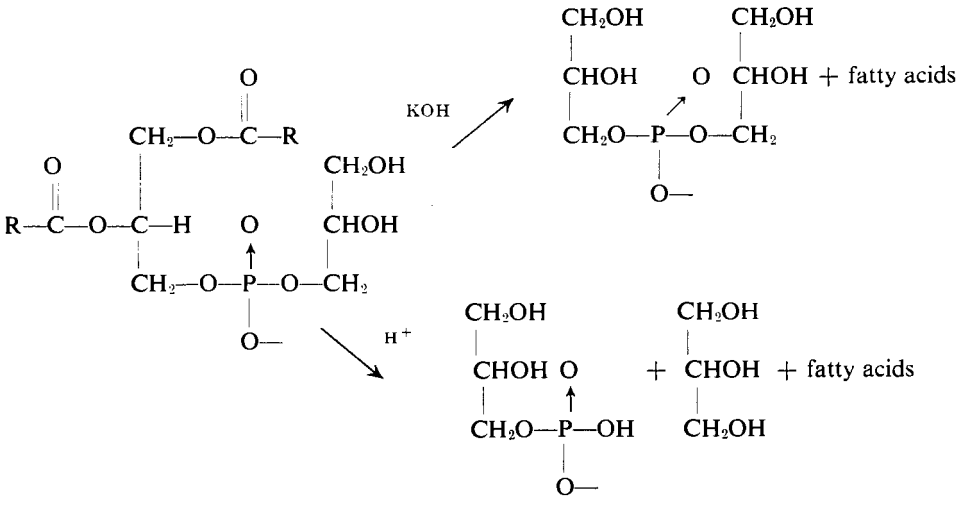
The lipid content of our memb ane preparation appears higher than the values reported by others (Shockman *et al.*, 1963; Ibbott and Abrams, 1964). This difference may be due to the inclusion of nonphosphatides in our data, or from differences in the growth medium or growth phase of the organisms. The absence of lipids in the cell wall fraction confirms the observations of Shockman *et al.* (1963). Chromatographic investigation of the lipids of the membrane and protoplasm revealed no significant qualitative or quantitative differences in lipid composition. The fatty acids of these lipids were not investigated in the present experiments.

Chromatography of the lipid extract on silicic acid–impregnated paper followed by specific staining procedures failed to show the presence of choline- serine, or ethanolamine-containing lipids indicating the absence of the usual types of phospholipids encountered among plant and animal tissues.

The glycosyl diglyceride structures for the carbohydrate-containing lipids were based on their chromatographic and staining characteristics and the products released by hydrolytic procedures. The reactions involved in the hydrolysis procedures are:



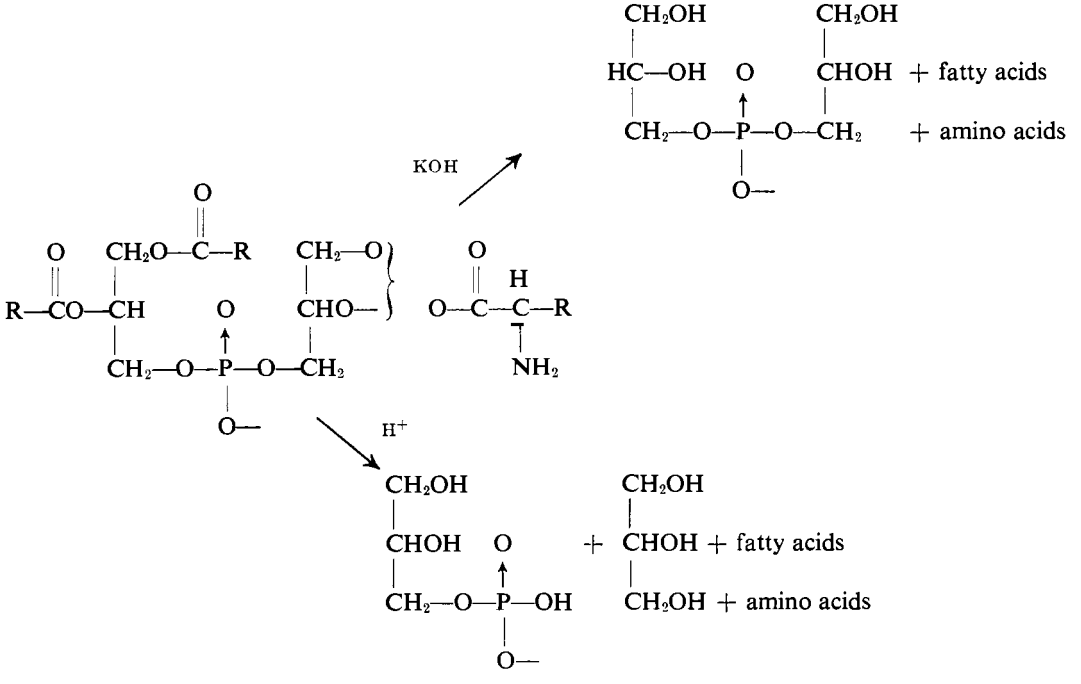
Although glucose and galactose were released in stoichiometric amounts from fraction B, it is not known whether they were combined as lactose in the intact lipid. Glycosyl diglycerides have been found in a number of microorganisms (Ferrari and Benson, 1961; MacFarlane, 1961, 1962a; Polonovski *et al.*, 1962),



SCHEME I

wheat flour lipids (Carter *et al.*, 1956), grasses (Weenink, 1961), brain (Steim and Benson, 1963), and photosynthetic tissues (Benson *et al.*, 1958; Zill and Harmon, 1962; Sastry and Kates, 1963). In photosynthetic tissues, Benson (1963) suggests that these neutral surfactant lipids stabilize the chloroplast membrane in addition to providing a physical pathway by which

high R_F value, the blue stain with Rhodamine 6G, and the negative reactions with ninhydrin and choline stains suggest a nonnitrogenous acidic phosphatide. A phosphatidyl glycerol type structure is indicated on the basis of the positive periodate-Schiff reaction. Although phosphatidic acid has been suggested to be the major phosphatide component of the membrane of



SCHEME II

hexoses are transported from the chloroplast to the aqueous regions of the cell. The function of this lipid class in nonphotosynthetic organisms, however, remains to be elucidated.

From the data presented, it is possible to postulate a phosphatidyl glycerol structure for the major phosphatide component (fraction D). The comparatively

S. faecalis (Shockman *et al.*, 1963) this structure is not consistent with our data. The R_F value of phosphatidic acid, 0.78 (Marinetti, 1962), is higher than that of spot 3 (Figure 2). In addition, a phosphatidic acid type structure would give a negative periodate-Schiff test. The water-soluble phosphate diester, glycerylphosphoryl glycerol, released on mild alkaline hydrolysis of fraction

D (Figure 4) and the release of glycerol and glycerol phosphate by acid hydrolysis further confirm a phosphatidyl glycerol type structure. The hydrolytic reactions are outlined in Scheme I.

The small amount of 1,3-diglycerylphosphoryl glycerol in the alkaline hydrolysate of fraction D indicates the presence of diphosphatidyl glycerol. These observations are somewhat different from those of Ibbott and Abrams (1964) who reported diphosphatidyl glyceride as the major phosphatide of the membrane of *S. faecalis*. This discrepancy may be a reflection of differences in growth conditions and physiological age of the culture. Previous work in this laboratory with *S. faecalis* (Vorbeck and Marinetti, 1964b) and studies with *Escherichia coli* (Kanfer and Kennedy, 1963) and *Staphlococcus aureus* (Houtsmuller and Van Deenen, 1964) indicate significant variation in the phosphatide composition during the various stages of growth.

The ninhydrin-positive phosphatide of fraction E has been tentatively identified as a lysine derivative of phosphatidyl glycerol. Small amounts of glycine and alanine also were found in the alkaline and acid hydrolysates of this fraction. These amino acids may be bound to the phosphatidyl glycerol molecule by an ester linkage at one or both of the available hydroxyl groups. The hydrolytic reactions are outlined in Scheme II. MacFarlane (1962b) has reported the occurrence of *O*-amino acid esters of phosphatidyl glycerol in *Clostridium welchii* and *S. aureus*, and Houtsmuller and Van Deenen (1964) have reported the occurrence of analogous components in other bacteria. Ikawa (1963) has found a lysine-containing phosphatide in *S. faecalis* but Shockman *et al.* (1963) did not find these lipids in the membranes of this same organism.

A comparison of the lipids extracted from stationary phase cells of *S. faecalis* (Vorbeck and Marinetti, 1965) and from exponential phase cells, as reported here, reveals significant increases in the amount of the phosphatidyl aminoacyl glycerol with increase in culture age. Using *S. aureus*, Houtsmuller and Van Deenen (1964) observed an accumulation of amino acid derivatives of phosphatidyl glycerol on lowering the *p*H of the medium. These authors suggest that the formation of a membraneous phospholipid containing a basic amino acid is a response of the organism to an increase in acidity of the surrounding medium.

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Components with Redox Potentiality in the "Neutral Lipid" Fraction from Beef Heart Mitochondria*

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ABSTRACT: Four components have been regularly observed in the neutral lipid fraction from mitochondria which show the ability to undergo reversible oxidation and reduction. The first is coenzyme Q. The second is found in the fraction which contains α-tocopherol. The third is a compound which shows higher R_F values on thin-layer chromatograms than any of the known redox components, but the identity of the compound is not clear.

The fourth shows low R_F on thin-layer chromatography and has the ultraviolet spectrum of a benzoquinone. The relation of these components to previous studies of the effects of lipids on the electron transport system is discussed.

The importance of lipid material in the function of the mitochondrion has been many times underlined. Most attention has been focused on the phospholipids as important constituents of biologial membranes. Besides this major class of lipid-soluble material, however, there exists a neutral lipid fraction in mitochondria whose composition has never been well defined.

With the name "neutral lipid fraction" we refer to the petroleum ether–soluble fraction after removal of the phospholipids. The best known component of this fraction is coenzyme Q_{10}, whose involvement in the mitochondrial respiratory chain as an electron carrier has been widely studied (Crane *et al.*, 1959a,b; Lester *et al.*, 1959; Hatefi *et al.*, 1959; De Bernard *et al.*, 1960; Szarkowska and Klingenberg, 1963; Chance 1965). α-Tocopherol is another constant component which has been well recognized and studied, whose intervention as a catalyst in the respiratory chain has been suggested many times (Bouman and Slater, 1957; Bouman *et al.*, 1958; Cowlishaw *et al.*, 1957; Nason and Lehman, 1956; Donaldson and Nason, 1957; Pollard and Bieri, 1960; Nason *et al.*, 1963). Its role is still not clear (Symposium on Vitamin E, 1962). Also vitamins of the K group (Martius, 1961) have been suggested many times as important components of mitochondria and proposals have been made that they are involved in electron transport or in energy coupling to respiration, but all the direct attempts to demonstrate their presence or their function in mitochondria have failed.

Recently it was possible to demonstrate that two undefined factors present in the neutral lipid fraction were necessary to restore completely the succinate oxidase system of mitochondria or submitochondrial particles which had been extracted with isooctane or acetone. The activity of these factors was in addition to the effect of coenzyme Q in restoration of the oxidase (Crane *et al.*, 1959a,b; Hendlin and Cook, 1960; Lester and Fleischer, 1961).

Some other suggestions of a role of lipid material in the mitochondrial function can be found in the literature (Marinetti *et al.*, 1957; Crawford *et al.*, 1959; Redfearn and King, 1964).

Because of the studies mentioned it seemed important to study the neutral lipid fraction of beef heart mitochondria. The most promising components would be

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