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Phospholipids from *Bacillus stearothermophilus*

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The lipids of *Bacillus stearothermophilus* strain 2184 were extracted with chloroform-methanol and separated into neutral lipid and three phospholipid fractions by chromatography on silicic acid columns. The phospholipids were identified by specific staining reactions on silicic acid-impregnated paper, by chromatography of alkaline and acid hydrolysis products, and by determination of acyl ester:glycerol:nitrogen:phosphorus molar ratios. The total extractable lipid was 8% of the dry weight of whole cells and consisted of 30 to 40% neutral lipid and 60 to 70% phospholipid. The phospholipid consisted of diphosphatidyl glycerol (23 to 42%), phosphatidyl glycerol (22 to 39%), and phosphatidyl ethanolamine (21 to 32%). The concentrations of diphosphatidyl glycerol and phosphatidyl glycerol were lower in 2-hr cells than in 4- and 8-hr cells. Whole cells were fractionated by sonic treatment and differential centrifugation. The total lipid content, expressed in per cent of dry weight of each fraction was: whole protoplasts, 10%; membrane fraction, 18%; 30,000 × *g* particulate fraction, 22%; and 105,000 × *g* particulate fraction, 26%. The relative phospholipid concentrations in each fraction were about the same. As had been previously reported, none of the phospholipid was stable to alkaline hydrolysis.

Bacillus stearothermophilus is an obligate thermophile with an optimal growth temperature between 60 and 65 C. Numerous investigations have established that proteins, highly purified enzymes (2, 22), and protein-synthesizing systems from thermophilic bacteria (9, 18) are more thermostable than comparable preparations from mesophilic bacteria. These findings suggest that the ability of thermophilic bacteria to grow at high temperatures is a result of the inherent thermostability of the individual cellular components and is not dependent on any stabilizing factor(s). There is, however, some evidence that certain membrane-bound enzymes may be stabilized by association with the cell membrane. Bubela and Holdsworth (5) reported that the amino acid-activating system from *B. stearothermophilus* was more heat stable when bound to the cell membrane than when released by sonic treatment, and suggested that the organization of the enzymes in the membrane could contribute to their thermostability. We have initiated an investigation of the lipid components of the cell membrane as a preliminary step to studies of membrane-enzyme associations. This paper reports on the major phospholipid components of *B. stearothermophilus* strain 2184.

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Previous reports of the phospholipids of *B. stearothermophilus* are conflicting and inconclusive. Georgi et al. (10) found that over 90% of the phospholipid component was stable to alkaline hydrolysis and suggested that the major phospholipid was sphingomyelin. This would be unique because sphingomyelin has not been found in any other bacterium. Long and Williams, on the other hand, were unable to detect sphingosine in the hydrolysis products (21). Also, they found no choline or ethanolamine, indicating that phosphatidyl choline and phosphatidyl ethanolamine were absent in *B. stearothermophilus*. We have identified the major phospholipid components of *B. stearothermophilus* strain 2184 as diphosphatidyl glycerol, phosphatidyl glycerol, and phosphatidyl ethanolamine.

MATERIALS AND METHODS

Growth of bacteria. *B. stearothermophilus* strain 2184 was grown at 60 C in a New Brunswick fermentor (New Brunswick Scientific Co., New Brunswick, N.J.). The culture medium contained 20 g of Trypticase (BBL), 1 g of glucose, 0.02 g of $MgSO_4 \cdot 7\ H_2O$, and 0.01 g of $FeCl_3$ per liter of distilled water. Foaming was controlled with Dow-Corning antifoam B. At the end of the incubation period (4 to 12 hr), the culture medium was poured into a 15-liter carboy containing sufficient crushed ice to lower the temperature to

about 10 C, and the cells were removed by centrifugation in a Sharples continuous-flow centrifuge.

Extraction of lipids. Wet cells or cell fractions were homogenized with methanol in a blender until a uniform dispersion was obtained. Chloroform was then added to a final concentration of 2:1 chloroform-methanol. The solvent-cell suspension (ratio of solvent to wet cell paste, about 40:1) was stirred at room temperature for 45 min and then filtered over a silicic acid pad on a medium-grade, sintered glass filter. The residue was washed consecutively with 100 ml each of chloroform, chloroform-methanol (2:1), and methanol. The combined extracts were evaporated to dryness, resuspended in chloroform-methanol (2:1), and washed with 0.29% NaCl, as described by Folch et al. (8). The washed lipid extract was evaporated to dryness, suspended in a small volume of chloroform, and stored at −15 C under nitrogen.

Column chromatography. The washed total extractable lipid (equivalent to 1 mg of phosphorus) was fractionated on silicic acid columns of the type described by Hirsch and Ahrens (14). The silicic acid (325 mesh, Bio-Rad Laboratories, Richmond, Calif.) was washed with chloroform-methanol (2:1) and chloroform and then dried and activated at 105 C for 4 hr. The activated silicic acid (10 to 20 g) was suspended in 50 ml of chloroform, poured into the column, and allowed to settle; it was then washed with 100 ml of chloroform. The lipid sample, containing 1 mg of lipid phosphorus, was dissolved in 10 ml of chloroform and placed on the column. Neutral lipid was eluted with chloroform. The phospholipids were eluted with the following step-wise gradient of chloroform-methanol: 20:1, 10:1, 7:1, 4:1, 3:2, 1:4, and methanol. Samples (10 ml) were collected and phosphorus was determined in each tube. In combining the separated fractions, the peak tubes were usually saved for further analysis.

Chromatography on silicic acid-impregnated paper. Silicic acid-impregnated papers were prepared as described by Marinetti (23). Lipids were chromatographed using a solvent system of diisobutylketone-acetic acid-water (40:20:3). The papers were examined for phosphorus, amino groups, choline, unsaturation, and vicinal hydroxyl groups (27).

Alkaline hydrolysis. The phospholipids were deacylated as described by Wintermans (28). Equal volumes (usually 0.5 ml) of 0.2 N methanolic KOH and a chloroform suspension of the phospholipid were mixed and held at 37 C for 15 min. The reaction was stopped by adding 0.2 ml of water and neutralizing with Dowex 50-8X ion-exchange resin (final *p*H, 6.5 to 7.0). Water and chloroform (1.0 ml each) were then added; the tube was shaken vigorously and centrifuged. The aqueous phase was carefully transferred with a capillary pipette and the volume was reduced under nitrogen. The water-soluble hydrolysis products were separated by two-dimensional chromatography on Whatman no. 1 paper (washed in 2 N acetic acid) by the use of 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. Phosphate esters were detected by spraying the paper with the following mixture: 10 ml of 70% (w/v) perchloric acid, 20 ml of 5 N HCl, 40 ml of 5% ammonium molybdate, and 130 ml of distilled water (6). The sprayed papers were irradiated with ultraviolet for about 15 min, and then stored in the dark. Spots usually developed within 2 or 3 hr.

Acid hydrolysis. For the determination of glycerol, the lipids were hydrolyzed by refluxing for 48 hr in 2 N HCl. For the recovery of ethanolamine, the lipid was hydrolyzed in 0.2 N methanolic HCl for 2 hr at 60 C. After extracting the free fatty acids with diethyl ether or chloroform, the water-soluble acid hydrolysis products were separated by ascending chromatography on Whatman no. 1 filter paper by use of one of the following solvent systems: phenol-water [100:38, w/v (4)]; butanol-acetic acid-water [77:6:17, v/v/v (20)]; isopropanol-ammonia-water [7:1:2, v/v/v (24)]. Amino groups were detected with ninhydrin, vicinal glycols by spraying with the acetyl-acetone reagent of Schwartz (25) after periodate oxidation, and phosphorus as described for the alkaline hydrolysis products (6).

Quantitative determination of phospholipid components. Two-dimensional chromatography of the alkaline hydrolysates proved the best way to separate and quantitate the different phospholipid components. The total lipid extract was placed on a silicic acid column and neutral lipid was eluted with chloroform. The total phospholipid was then eluted with chloroform-methanol (2:1). Recovery of lipid phosphorus from the column was nearly 100%. The mixed phospholipid was deacylated and water-soluble phosphate esters were separated by two-dimensional chromatography as described previously. After development, the papers were stained for phosphorus groups. The individual spots (in addition to a 0.5-cm border around each spot) were cut out, weighed, and digested at 190 C with 70% perchloric acid (6). A paper blank, cut from the center of the chromatogram, served as a control. The recovery of total phosphorus from 10 papers chromatographed with the same lipid sample was 96.6 ± 3%. The percentage of phosphorus in the individual spots agreed within 3% for the 10 papers.

Analytical procedures. Phosphorus was determined, as described by Bartlet (3), after digestion in 10 N H_2SO_4 or 70% perchloric acid. Nitrogen was determined by the Kjeldahl-Nessler procedure (19), ester groups by the procedure of Snyder and Stephens (26), and glycerol by the method of Hanahan and Olley (13) by the use of monomethylol dimethyl hydantoin as a standard. Lipid content was determined gravimetrically after drying to constant weight in a stream of nitrogen.

Fractionation of B. Stearothermophilus. Protoplasts were prepared by suspending freshly harvested and washed cells from a 4-hr culture in a solution containing 0.15 M NaCl, 0.05 M Tris-hydrochloride buffer (*p*H 7.5), and 0.15% lysozyme. Protoplast formation was followed with a phase-contrast microscope. After 30 min at room temperature, the suspension was centrifuged and the protoplasts were carefully suspended in 0.15 M NaCl containing 0.002 M $MgSO_4$. (Under these conditions, the destruction of protoplasts, as observed by phase microscopy, was less than 5%, provided that one observes cautious handling and

careful pipetting.) The membrane fraction was prepared by lysing the protoplasts in distilled water. The viscous suspension of lysed protoplasts was stirred at 4 C for 30 min and then centrifuged at 30,000 × *g* for 30 min. The pale yellow supernatant fluid was discarded, and the membranes were washed twice by suspending the pellet in distilled water and centrifuging at 30,000 × *g* for 30 min. A distilled-water suspension of membranes was further fractionated by sonic treatment (five 30-sec pulses in a Branson Sonifier model L575) and centrifugation at 30,000 × *g* for 30 min followed by centrifugation of the 30,000 × *g* supernatant fraction at 105,000 × *g* for 4 hr (Spinco model L-40 rotor). The 30,000 × *g* particulate fraction consisted of a greyish-white material covered by a layer of light pink material. The 105,000 × *g* particulate fraction was bright red and contained the electron transport particles described previously (7).

Materials. Diphosphatidyl glycerol (cardiolipin) and phosphatidyl ethanolamine were purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Phosphatidyl glycerol was prepared from spinach, as described by Halverkate and van Deenen (11). All solvents were reagent grade and were not redistilled before use.

RESULTS AND DISCUSSION

The distribution of lipid phosphorus after mild alkaline hydrolysis is shown in Table 1. After 20 min, all of the phosphorus was found in the water phase. *B. stearothermophilus* contains no phospholipid stable to alkaline hydrolysis, as previously reported (10).

Characterization of the phospholipid. Lipid extracts were fractionated on silicic acid columns. A typical elution pattern obtained with an extract from a 2-hr culture is shown in Fig. 1. Similar elution patterns were obtained with lipids extracted from 4-, 6-, 8-, 10-, and 12-hr cultures. In the experiment shown, fraction A contained 48%, fraction B contained 9%, and a combination of fractions C, D, and E contained 42% of the total lipid phosphorus. The failure to elute any lipid phosphorus at ratios of chloroform-methanol higher than 20:1 (90:1, 50:1, and 30:1 were tried) suggested that no phosphatidic acid was present in the lipid extract. Also, the fact that less than 5% of the total phosphorus eluted at ratios lower than 7:1 suggested that if phosphatidyl choline or phosphatidyl inositol were present, they were minor components.

TABLE 1. *Phosphorus distribution following alkaline hydrolysis of total lipid*[a]

Hydrolysis time	Phosphorus	Phosphorus distribution	
		Chloroform phase	Water phase
min	*μg*	*μg*	*μg*
0	76.8	74.6	2.7
10	76.8	12.2	62.8
20	76.8	<0.5	76.5
40	76.8	<0.5	76.5
60	76.8	<0.5	75.4

[a] Total lipid was hydrolyzed in 0.1 N methanolic NaOH (1 ml) at room temperature. The reaction was stopped by the addition of water, chloroform, and 1 N HCl.

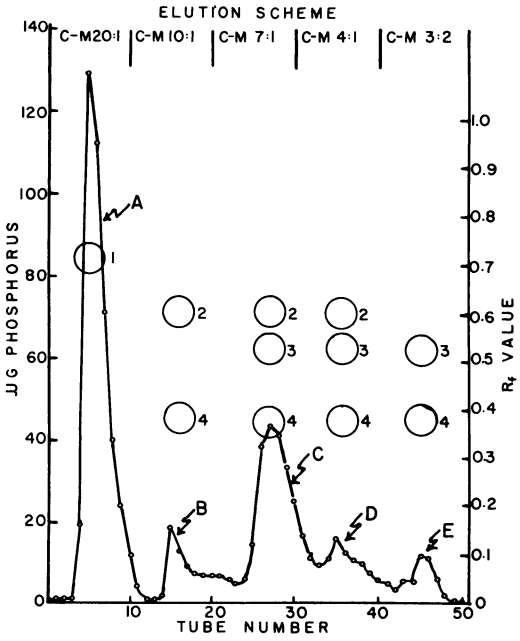

FIG. 1. *Silicic acid column chromatography of phospholipid from a 2-hr culture of B. stearothermophilus. The phospholipids were eluted with 100 ml of each of the chloroform-methanol mixtures shown at the top of the figure. A, B, C, D, and E indicate the major peaks of phospholipid eluted with chloroform-methanol. Samples were removed from tubes 5, 15, 27, 34, and 45 and chromatographed on silicic acid-impregnated paper. Spots 1, 2, 3, and 4 correspond to the various phospholipid components of each fraction.*

The further separation of the phospholipids found in the peak tubes was performed on silicic acid-impregnated paper. The R_F values and number of spots obtained with each fraction are illustrated in Fig. 1. Fraction A contained only one phospholipid component, whereas fractions B, C, D, and E contained at least two phospholipid components. The different staining reactions for each of the spots are reported in Table 2.

When the total lipid extract was chromatographed on silicic acid-impregnated paper, only spots 1, 2, and 3 were detected. Spot 4 was observed only in preparations which had been on the

TABLE 2. *Staining reactions and R_F values on silicic acid-impregnated paper*[a]

Spot	R_F	Phosphate	Amino groups	Unsaturation	Vicinal hydroxyls
1	0.73	+	−	−	−
2	0.61	+	−	−	+
3	0.53	+	+	+	−
4	0.38	+	−	−	−
DiPG[b]	0.75	+	−	+	−
PG[c]	0.60	+	−	+	+
PE[d]	0.53	+	+	+	−

[a] Ascending chromatography using diisobutyl ketone-acetic acid-water (40:20:3). Papers were stained, as described by Marinetti (23), by the use of acid molybdate for phosphate, ninhydrin for amino groups, $KMnO_4$ for unsaturation, and the periodate-Schiff reagents for vicinal hydroxyls (27).

[b] Diphosphatidyl glycerol (cardiolipin).

[c] Phosphatidyl glycerol.

[d] Phosphatidyl ethanolamine.

silicic acid columns for extended periods of times. This component was assumed to be a breakdown product, and was not analyzed further. (This was propably a lysophosphatide form of diphosphatidyl glycerol, since a small amount of diglyceryl-phosphoryl glycerol could be detected in the alkaline hydrolysis product of all fractions from an initial column separation.) When compared to authentic samples of phospholipid, spot 1 corresponded to diphosphatidyl glycerol (cardiolipin), spot 2 corresponded to phosphatidyl glycerol, and spot 3 corresponded to phosphatidyl ethanolamine (Table 2).

Identification of hydrolysis products. The separation and identification of alkaline hydrolysis products was best accomplished by two-dimensional ascending chromatography 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 (4). A typical chromatogram obtained by chromatographing the total phospholipid hydrolysate is shown in Fig. 2. Four phosphorus-containing spots were obtained. These correspond to 1,3-diglycerylphosphoryl glycerol (GPGPG), glycerylphosphoryl glycerol (GPG), cyclic glyceryl-phosphate (cycGP), and glycerylphosphoryl ethanolamine (GPE). When stained with ninhydrin, only one amino nitrogen spot was obtained, and this corresponded to GPE. The cycGP is a hydrolysis product of GPG and was included with the GPG spot for the quantitative determination of phosphatidyl glycerol (Fig. 2).

Deacylation of fraction A (Fig. 1) yielded only GPGPG (Table 3). The molar ratio of acyl ester to amino nitrogen to glycerol to phosphorus was 2.06:0.01:1.51:1. The expected ratio for diphosphatidyl glycerol would be 2:0:1.5:1. The hydrolysate of fraction B from an initial column fractionation consisted mainly of GPG and cycGP with a trace of GPGPG. The phosphatidyl glycerol was purfied by combining the 10:1 eluates from several samples and rechromatographing on silicic acid columns. The lipid from the peak tubes of the 10:1 fraction of the second separation yielded only GPG and cycGP on hydrolysis (Table 3). The ratio of acyl ester to amino nitrogen to glycerol to phosphorus was 2.18:<0.01:2.22:1 for the rechromatographed fraction B. The expected ratio for phosphatidyl glycerol would be 2:0:2:1. Fraction C from an initial column fractionation yielded GPG, cycGP, and GPE as the major hydrolysis products, as well as a trace of GPGPG. The phosphatidyl ethanolamine was purified by combining the 7:1 eluates from several samples and rechromatographing on silicic acid columns. The phospholipid was eluted with chloroform-methanol (10:1) followed by chloroform-methanol (7:1). The 7:1 eluates were collected and rechromatographed in this manner until only GPE could be detected in the hydrolysis products from the peak tubes (Table 3). This usually required three separations on the silicic acid columns. The ratio of acyl ester to amino nitrogen to glycerol to phosphorus was 2.01:0.94:1.21:1 for the purified fraction C. The expected ratios for phosphatidyl ethanolamine would be 2:1:1:1.

The products obtained after hydrolysis in 0.2 M methanolic HCl at 60 C for 2 hr are shown in Table 4. Fraction A gave glycerol and α-glyceryl phosphate. Only one phosphorus-containing spot was obtained and this corresponded to α-glyceryl phosphate. The same products were obtained from fraction B. Fraction C contained α-glyceryl phosphate, ethanolamine, and usually a trace of GPE. When the total mixed phospholipid was hydrolyzed, glycerol, α glyceryl phosphate, and ethanolamine were obtained.

Concentration of phospholipids after incubation periods of 2, 4, and 8 hr. Cultures of *B. stearothermophilus* were grown and harvested after incubation for 2, 4, and 8 hr. The lipid was extracted and the concentrations of diphosphatidyl glycerol, phosphatidyl glycerol, and phosphatidyl ethanolamine were determined by chromatography of the alkaline hydrolysis products.

All of the phospholipid preparations obtained from the 2-, 4-, and 8-hr cells contained the same four water-soluble deacylation products (Fig. 2). Staining of the chromatograms with ninhydrin

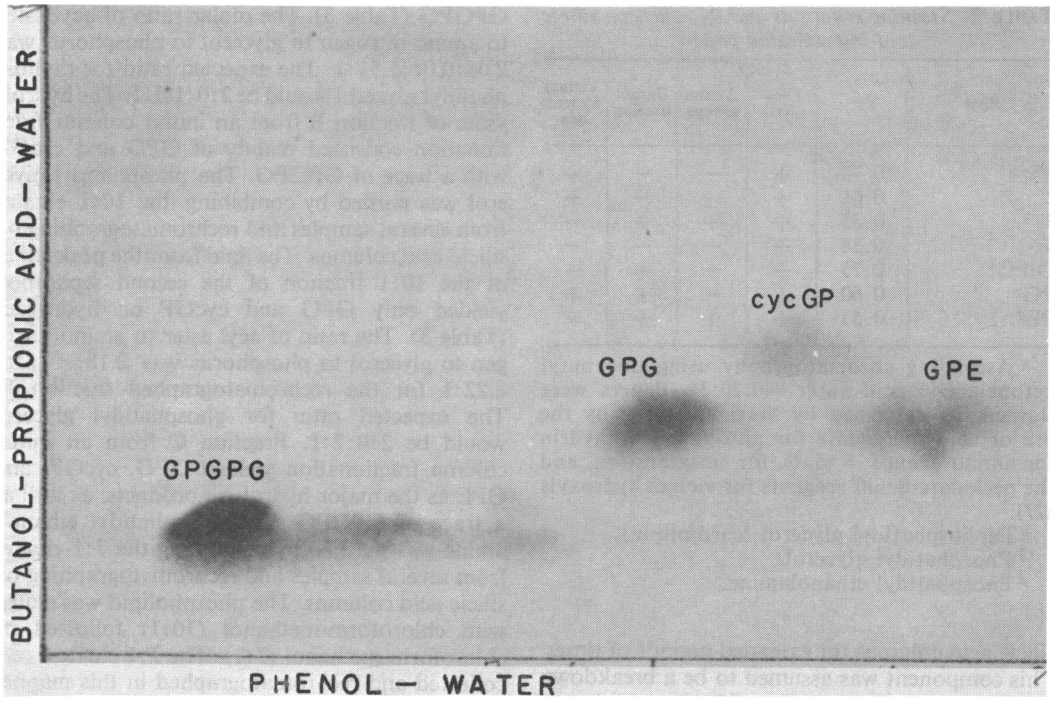

FIG. 2. *Two-dimensional chromatography of the water-soluble alkaline hydrolysis products. The total mixed phospholipid was deacylated, and the water soluble products were separated by ascending chromatography.*

showed only one spot corresponding to GPE. All attempts to obtain evidence for amino acid esters of phosphatidyl glycerol were negative. Lowering the *p*H value of the medium to about 2.0 immediately before harvesting, or 10 min before harvesting, made no difference in the pattern of lipid extracted (15).

The analysis of the lipid extracted from cells harvested after 2, 4, and 8 hr is shown in Table 5. The main difference observed was an increase in the diphosphatidyl glycerol and phosphatidyl glycerol content and a decrease in the phosphatidyl ethanolamine content between 2 and 4 hr of incubation. This change in phospholipid composition was accompanied by a change in *p*H value of the growth medium. The initial *p*H of 6.8 dropped steadily to *p*H 5.8 at 2.5 hr, and then increased steadily to *p*H 8.0 at 6 hr, where it remained constant for the rest of the incubation period. The lipid composition of the 4- and 8-hr cells were similar except that the cells harvested at 8 hr contained more phosphatidyl ethanolamine.

We consider it doubtful that the change in *p*H value had any direct effect on the phospholipid composition. Both changes probably result from a change in the metabolism of the cells. The effect of changes in the activity of various enzymes (especially membrane-bound enzymes) on the phospholipid composition is currently under investigation.

The total lipid content of *B. stearothermophilus* (about 8%) was higher than most gram-positive bacteria (about 1 to 3%) and more comparable to the gram-negative bacteria which have been examined [about 5 to 10% (17)]. Although phosphatidyl ethanolamine is absent from most gram-positive bacteria, it has been reported for several species of *Bacillus* (16).

Cellular distribution of the phospholipid. *B. stearothermophilus* was fractionated and the phospholipid composition of each fraction was determined. The yield of each fraction for the experiment shown in Table 6 was: protoplasts, 0.87 g; membranes, 0.24 g; 30,000 × *g* particulate fraction, 0.02 g; and 105,000 × *g* particulate fraction, 0.14 g per gram (dry weight) of whole cells. It is apparent from the data in Table 6 that the phospholipid distribution was fairly uniform in all fractions. The percentage of total lipid in the protoplasts was slightly higher than in the whole cells (Table 4). The phospholipid represented about 75% of total lipid of the protoplasts compared to 65% for whole cells. Apparently some of the neutral lipid is released during protoplast formation. The protoplast fraction contained 97.5 ± 2% of the total phospholipid of the whole cells.

The membrane fraction contained 91.3 ± 3% of the total phospholipid of the whole cells. This is in accord with other reports that the phospholipid is almost exclusively associated with membranes (12). The 30,000 × *g* particulate fraction contained 8% of the total phospholipid, and the 105,000 × *g* fraction contained 52% of the total phospholipid of the whole cells. The percentage of total lipid in the membrane fractions agrees with reported values for other membrane systems (17). The yield of material which sedimented at 30,000 × *g* and 105,000 × *g* (from the sonic-treated membrane suspension) varied considerably from sample to sample; however, the relative distribution of the phospholipid was the same for all samples. Abram has shown by electron micrography that the membrane of *B. stearothermophilus* consists of a base unit to which particles are attached (1). If the 30,000 × *g* material and the 105,000 × *g* material represent different components of the membrane, then it appears that the phospholipid is evenly distributed throughout the cell membrane. It will be interesting to determine the phospholipid composition of various enzyme systems isolated from the membrane. Preliminary evidence suggests that phospholipid is uniformly distributed throughout the membrane.

TABLE 3. *Paper chromatography of alkaline hydrolysis products*[a]

Component	Solvent system[b]	
	PW	BPW
	R_F	R_F
GPGPG[c]	0.16	0.09
GPG[d]	0.39	0.17
GPE[e]	0.62	0.17
Fraction A[f]	0.16	0.06
Fraction B	0.38	0.17
Fraction C	0.62	0.17

[a] Ascending chromatography on Whatman no. 1 paper. Phosphate esters were detected with the acid molybdate spray (6).

[b] PW, phenol-water (100:38, w/v); BPW, butanol-propionic acid-water (142:71:100, v/v/v).

[c] Prepared by deacylation of diphosphatidyl glycerol (cardiolipin).

[d] Prepared by deacylation of phosphatidyl glycerol extracted from spinach.

[e] Prepared by deacylation of phosphatidyl ethanolamine.

[f] Fractions were obtained by repeated chromatography on silicic acid columns.

TABLE 4. *Paper chromatography of acid hydrolysis products*[a]

Component	Solvent systems[b]	
	BAW	IPAW
	R_F	R_F
Glycerol	0.51	0.66
α-Glyceryl phosphate	0.17	0.11
Ethanolamine	0.13	0.55
Fraction A[c]	0.51	0.66
	0.17	0.11
Fraction B	0.51	0.66
	0.17	0.11
Fraction C	0.17	0.11
	0.13	0.55

[a] Ascending paper chromatography of the water-soluble products obtained by hydrolysis of the isolated fractions in 0.2 M HCl for 2 hr at 60 C. Papers were stained with the acetylacetone reagent (25), after periodate oxidation for vicinal hydroxyls, or with ninhydrin for amino nitrogen.

[b] BAW, butanol-acetic acid-water (77:6:17, v/v/v); IPAW, isopropanol-ammonium hydroxide-water (7:1:2, v/v/v).

[c] Fractions were obtained after repeated chromatography on silicic acid columns.

TABLE 5. *Lipid analysis of cells harvested after incubation for 2, 4, and 8 hr*[a]

Incubation time	Total lipid; per cent of cell (dry weight)	Phospholipid; per cent of total lipid	DiPG[b]	PG[c]	PE[d]
Hr			*μmole*	*μmole*	*μmole*
2	7.25	61	9.4	8.8	12.9
4	8.50	62	14.9	14.6	7.5
8	8.30	69	14.0	16.3	10.9

[a] Phospholipid concentrations were determined by two-dimensional chromatography of the alkaline hydrolysis products.

[b] Diphosphatidyl glycerol was calculated on the basis of two molecules of phosphorus per molecule of diphosphatidyl glycerol and is expressed as micromoles of phospholipid per gram of cells (dry weight).

[c] Phosphatidyl glycerol, expressed as micromoles of phospholipid per gram of cells (dry weight).

[d] Phosphatidyl ethanolamine, expressed as micromoles of phospholipid per gram of cells (dry weight).

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LITERATURE CITED

1. Abram, D. 1965. Electron microscope observations of intact cells, protoplasts and the cytoplasmic membrane of *Bacillus stearothermophilus*. J. Bacteriol. 89:855–873.

TABLE 6. *Lipid analysis of cell fractions*[a]

Cell fraction	Total lipid; per cent of fraction (dry weight)	Phospholipid; per cent of total lipid	Percent of total phospholipid[b]		
			DiPG	PG	PE
Protoplasts	9.8	75.2	48.8	24.3	23.4
Membranes	18.3	77.2	49.4	28.0	19.4
Particulate fraction (30,000 × *g*)	22.0	78.8	46.0	27.3	25.2
Particulate fraction (105,000 × *g*)	26.4	77.2	46.0	23.2	20.6

[a] Phospholipid concentrations were determined by chromatography of the alkaline hydrolysis products. Fractions were prepared as previously described.

[b] DiPG, diphosphatidyl glycerol; PG, phosphatidyl glycerol; PE, phosphatidyl ethanolamine.

2. Amelunxen, R. E. 1966. Crystallization of thermostable glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*. Biochim. Biophys. Acta 122:175–181.
3. Bartlet, G. R. 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234:466–468.
4. Benson, A. A., and E. H. Strickland. 1960. Plant phospholipids. III. Identification of diphosphatidyl glycerol. Biochim. Biophys. Acta 41:328–333.
5. Bubela, B., and E. S. Holdsworth. 1966. Protein synthesis in *Bacillus stearothermophilus*. Biochim. Biophys. Acta 123:376–389.
6. Dawson, R. M. C., N. Hemington, and J. G. Davenport. 1962. Improvements in the method of determining individual phospholipids in a complex mixture by successive chemical hydrolyses. Biochem. J. 84:497–501.
7. Downey, R. J., C. E. Georgi, and W. E. Militzer. 1961. Electron transport particles from *Bacillus stearothermophilus*. J. Bacteriol. 83:1140–1146.
8. Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497–509.
9. Friedman, S. M. 1968. Protein-synthesizing machinery of thermophilic bacteria. Bacteriol. Rev. 32:27–38.
10. Georgi, C. E., W. E. Militzer, and T. S. Decker. 1955. The organelle nature of a particle isolated from *Bacillus stearothermophilus*. J. Bacteriol. 70:716–725.
11. Halverkate, R., and L. L. M. van Deenen. 1963. Isolation of phosphatidyl glycerol from spinach leaves. Biochem. J. 88:42–43.
12. Hanahan, E. J. 1960. Lipid chemistry, p. 1–10. John Wiley & Sons, Inc., New York.
13. Hanahan, E. J., and J. N. Olley. 1958. Chemical nature of monophosphoinositides. J. Biol. Chem. 231:813–828.
14. Hirsch, J., and E. H. Ahrens, Jr. 1958. The separation of complex lipid mixtures by the use of silicic acid chromatography. J. Biol. Chem. 233:311–320.
15. Houtsmuller, U. M. T., and L. L. M. van Deenen. 1964. On the accumulation of amino acid derivatives of phosphatidyl glycerol in bacteria. Biochim. Biophys. Acta 84:96–98.
16. Ikawa, M. 1967. Bacterial phosphatides and natural relationships. Bacteriol. Rev. 31:54–64.
17. Kates, M. 1964. Bacterial lipids, p. 17–90. *In* R. Raoletti and D. Kritchevsky (ed.), Advances in lipid research, vol. 2. Academic Press, Inc., New York.
18. Koffler, H. 1957. Protoplasmic differences between mesophiles and thermophiles. Bacteriol. Rev. 21:227–240.
19. Lang, C. A. 1958. Simple microdetermination of Kjeldahl nitrogen in biological materials. Anal. Chem. 30:1692–1694.
20. Lederer, E., and M. Lederer. 1957. Chromatography: A review of principles and applications, p. 208. Elsevier Publishing Co., New York.
21. Long, S. K., and O. B. Williams. 1960. Lipids of *Bacillus stearothermophilus*. J. Bacteriol. 79:629–637.
22. Manning, G. B., L. L. Campbell, and R. J. Foster. 1961. Thermostable α-amylase of *Bacillus stearothermophilus*. II. Physical properties and molecular weight. J. Biol. Chem. 236:2958–2961.
23. Marinetti, G. V. 1962. Chromatographic separation, identification, and analysis of phosphatides. J. Lipid Res. 3:1–20.
24. Plackett, P. 1964. A synthesis of 1,3-di-*O*-(glycerol-3-phosphoryl) glycerol. Australian J. Chem. 17:101–108.
25. Schwartz, D. P. 1958. Specific identification of hydroxyamino acids on paper chromatograms of protein hydrolysates. Anal. Chem. 30:1855–1856.
26. Snyder, F., and N. Stephens. 1959. A simplified spectrophotometric determination of ester groups in lipids. Biochim. Biophys. Acta 34:244–245.
27. Vorbeck, M. L., and G. V. Marinetti. 1965. Intracellular distribution and characterization of lipids of *Streptococcus faecalis* (ATCC 9790). Biochemistry 4:296–305.
28. Wintermans, J. F. G. M. 1960. Concentrations of phosphatides and glycolipids in leaves and chloroplasts. Biochim. Biophys. Acta 44:49–54.