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Membrane Phospholipid Asymmetry in *Bacillus amyloliquefaciens*

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The phospholipid distribution in the membrane of *Bacillus amyloliquefaciens* was studied by using phospholipase C (*B. cereus*), phospholipase A_2 (*Crotalus*), and the nonpenetrating chemical probe trinitrobenzenesulfonic acid. After treatment of intact protoplasts of *B. amyloliquefaciens* with either phospholipase, about 70% of total membrane phospholipid was hydrolyzed; specifically, about 90, 90, and 30% of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin, respectively. Under these conditions, protoplasts remained intact and sealed. However, when protoplasts that were permeabilized by cold-shock treatment were incubated with either of the phospholipases, up to 80% of cardiolipin was hydrolyzed and phosphatidylglycerol and phosphatidylethanolamine were hydrolyzed virtually to completion. In intact cells, 92% of the phosphatidylethanolamine could be labeled with trinitrobenzenesulfonic acid under conditions in which the reagent did not penetrate the membrane to any significant extent. These results indicate that 70% of total phospholipid of this bacillus exists in the outer half of the bilayer. The distribution of phosphatidylethanolamine in this bilayer is highly asymmetric with it being located predominantly in the outer half. The results with phospholipases suggest that the distributions of cardiolipin and phosphatidylglycerol are also asymmetric but independent confirmation of this is required.

Specific phospholipases and chemical labeling reagents have been used to examine the phospholipid distribution in the bilayer of many biological membranes. Studies, particularly with the erythrocyte membrane (7, 20–22), clearly established an asymmetrical distribution of phospholipids between the two halves of the bilayer. Other workers have proposed asymmetric distributions of phospholipids in other animal membranes (11, 19), bacteriophages (17), and viral membranes (6, 15).

The phospholipid distribution in some gram-positive bacteria has also been investigated. Barsukov et al. (1) reported that in the cytoplasmic membrane of *Micrococcus lysodeikticus* there was an asymmetric distribution of phospholipids, whereas Rothman and Kennedy (13) concluded that 33% of the phosphatidylethanolamine (PE) was located in the outer monolayer of the cytoplasmic membrane of *Bacillus megaterium*. Recently, Bishop et al. (3) reported on studies dealing with the phospholipid arrangement in *B. subtilis* Marburg and found that at least 60% of the PE is located in the outer monolayer.

In the present work we show that the phospholipids in the membrane of *B. amyloliquefaciens* are asymmetrically arranged, with 70% of the total phospholipid existing in the outer monolayer. Although the phospholipase results alone do not allow an unequivocal distribution of phosphatidylglycerol (PG) and cardiolipin (CLP) to be made, it is clear that PE is localized predominantly in the outer half of the bilayer. Of the total PE, 90% can be hydrolyzed by phospholipases or labeled with trinitrobenzenesulfonic acid (TNBS). This distribution of PE is markedly different from that found in the cytoplasmic membrane of *B. megaterium* (13).

MATERIALS AND METHODS

Growth of organism. A mutant strain of *B. amyloliquefaciens* that does not secrete "surfactin" (16) was grown from a spore inoculum in a medium described previously (4). Flasks were incubated at 30°C in an orbital shaking incubator (Paton model 461), oscillating at 300 rpm. The cells were harvested after 18 h of growth when they had reached late logarithmic phase (absorbance at 600 nm [A_{600}] = 3.6).

When cells were to be uniformly labeled with ^{32}P, 20 μCi of ^{32}P-labeled inorganic phosphate per ml was added to the growth medium at the time of inoculation. The growth medium contained 34 mM cold phosphate as carrier.

Preparation of protoplasts. Cells were harvested, washed once in 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.3) and resuspended in their initial volume of protoplast medium. This medium contained Tris (25 mM), $(NH_4)_2HPO_4$ (3.8 mM), KCl (5 mM), $CaCl_2$ (0.125 mM), $MgSO_4$ (5

mM), 0.25 ml of the trace metal solution described by Both et al. (4) per liter, 1% (wt/vol) maltose, 0.025% (wt/vol) Casamino Acids (Difco), and 22% (wt/vol) sucrose, adjusted to pH 7.3 with HCl. The cells were then gently swirled at 30°C in an orbital shaking water bath (Paton model OW1412) in the presence of 133 μg of lysozyme (Sigma) per ml. Protoplast formation was complete within 60 min, as judged by phase contrast microscopy.

Cold-shock permeablization of protoplasts. To make protoplasts leaky, cells were incubated with lysozyme as before, and then when formation was complete, the protoplasts were centrifuged (4,000 × *g*, 5 min). The protoplast pellet was taken up in 0.5 ml of protoplast medium at 30°C and added slowly to 9.5 ml of ice-cold protoplast medium.

Incubation with phospholipase A_2 and C. Protoplasts were resuspended in fresh protoplast medium such that a 1-ml portion contained about 100 μg of phospholipid. These portions were incubated at 37°C with either 4 IU of phospholipase A_2 (*Crotalus*) (purchased as a solution in 50% glycerol [wt/vol] from Boehringer Mannheim Corp.) or 30 IU of phospholipase C (*B. cereus*) (type V, purchased as a suspension in 3.2 M ammonium sulfate from Sigma Chemical Co.). Control protoplasts were incubated with the addition of a volume of 50% glycerol (vol/vol) or 3.2 M $(NH_4)_2SO_4$ equal to the volume of enzyme added to the other portions for phospholipase A_2 and C, respectively. When phospholipase A_2 was used, the incubation medium was supplemented with 10 μl of 0.1 M $CaCl_2$. At the end of the incubation period, the enzymes were inhibited by addition of 100 μl of 1 M EDTA before lipid extraction.

When intact cells or membrane vesicles were incubated with phospholipase C, they were suspended in 50 mM Tris-hydrochloride, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ (pH 7.3), such that a 1-ml portion contained about 100 μg of phospholipid. These portions were then incubated with 30 IU of phospholipase C as described above.

Extraction of lipids. Lipids were extracted by the method of Kates (10), except that the final lipid extract was dehydrated with Na_2SO_4 (anhydrous) rather than with benzene, before being dried under a stream of nitrogen. This procedure involved two extraction steps with chloroform-methanol (1:2 vol/vol), 99% of total phospholipid being extracted by the first. However, to obtain complete extraction of cardiolipin from intact cells, it was necessary to first disrupt the cells in an Aminco French pressure cell operated at 15,000 lb/in^4.

Thin-layer chromatography and quantitation of phospholipids. Phospholipids were separated by two-dimensional thin-layer chromatography on plastic-backed silica gel plates (Merck, Kieselgel 60 F254). Samples (10 μl) of total lipid extract in $CHCl_3$ were loaded on to plates (10 by 10 cm) which after drying under N_2 were developed in chloroform-methanol-water (65:25:4, vol/vol) in the first dimension, then dried under N_2 and developed in chloroform-methanol-acetic acid (65:25:4, vol/vol) in the second dimension. The location of radioactive phospholipid spots was determined by overnight autoradiography. The spots were then scraped off and counted by liquid scintillation. In all cases, the recovery of ^{32}P counts from the chromatograms was greater than 95%. Phospholipid species were identified by comparing their migration with that of phospholipid standards purchased from Sigma Chemical Co. and Applied Science Laboratories Inc.

Assay for leakage of intracellular ribonuclease inhibitor from protoplasts. Protoplasts were incubated with or without added phospholipases and then centrifuged (4,000 × *g*, 5 min). A fixed amount of *B. amyloliquefaciens* ribonuclease was added, and the mixture was then assayed for ribonuclease activity by the method of Coleman and Elliott (5). The amount of inhibitor present was directly proportional to the number of units of ribonuclease inactivated. The total amount of ribonuclease inhibitor inside the protoplasts was determined by lysing them in an Aminco French pressure cell at 15,000 lb/in^2, centrifuging at 4,000 × *g* for 5 min, and then assaying the supernatant fraction for ribonuclease inhibitor as described above.

Labeling of Cells with TNBS. TNBS was purchased from Sigma Chemical Co. and was used without further purification. The procedure used is essentially the same as that of Rothman and Kennedy (13). Cells (5 ml) grown in ^{32}P were harvested, washed once in 0.1 M KCl, containing 50 mM potassium phosphate (pH 7.9), and taken up in 5 ml of the same buffer. The cells were slowly cooled to 4°C to avoid cold shock (18), and 28 mM TNBS in 5% (wt/vol) $NaHCO_3$ (pH 8.5) was added to the desired final concentration of TNBS (3 mM for most experiments). The pH of the suspension was maintained at pH 8.1. The cells were incubated at 4°C in an orbital shaking water bath. Samples (0.5 ml) were withdrawn at intervals, and the reaction was stopped by addition of 100 μl of ice-cold 30% (wt/vol) trichloroacetic acid and 25 μl of a 5-mg/ml solution of bovine serum albumin (BSA). After 5 min on ice, the precipitate was collected by centrifugation, taken up in 1 ml of 0.1 M KCl–0.1 N HCl, and then the lipid was extracted as described above. The PE and trinitrophenyl-PE spots were separated by two-dimensional thin-layer chromatography and quantitated as described above. PE and trinitrophenyl-PE were well separated by this technique, their respective R_f values being 0.54 and 0.61 for the first dimension and 0.33 and 0.88 for the second dimension.

Assay for entry of TNBS into cells. The extent of entry of TNBS into cells at 4°C was estimated by measuring the extent of labeling of a crude cytoplasmic protein fraction. Labeling of protein was detected by the yellow color of the trinitrophenyl group. Two approaches were used. Portions (10 ml) of cells were incubated at 4°C with 3 mM TNBS as before, and after labeling for 4 h the cells were washed with 40 ml of 50 mM Tris-hydrochloride containing 50 mM KCl and 20 mM 2-mercaptoethanol (pH 8.5) and resuspended in 10 ml of 50 mM potassium phosphate containing 100 mM KCl (pH 7.0). The cells were then lysed in a French pressure cell at 15,000 lb/in^2 and centrifuged (105,000 × *g*, 60 min). Supernatant protein was precipitated with 1.2 ml of ice-cold 30% (wt/vol) trichloroacetic acid and 0.4 ml of a 5-mg/ml solution of BSA. The pellet was then collected by centrifugation, taken up in 10 ml of 1 M Tris-free base containing 5% (wt/vol) sodium dodecyl sulfate, and the A_{410} was measured in a Hitachi model 101 spectrophotometer.

The absorbance was compared with that of the trinitrophenylated crude cytoplasmic protein fraction of cells that were lysed in the French pressure cell before 4 h of labeling with TNBS at 4°C. In each case the A_{410} of the crude cytoplasmic protein fractions from control cells that were not trinitrophenylated was subtracted from the readings.

This procedure, however, does not separate peripheral protein external to the cytoplasmic membrane or membrane protein shed from the bilayer during French pressure cell treatment from the crude cytoplasmic protein fraction, and, therefore, a second procedure was employed. Portions (10 ml) of cells were labeled with TNBS and washed at the end of the incubation period as before. Then, rather than being lysed in the French pressure cell, the cells were suspended in protoplast medium and incubated with 133 μg of lysozyme per ml at 30°C for 60 min, yielding protoplasts. These protoplasts were then washed in protoplast medium and lysed osmotically. The lysate was centrifuged, and the extent of labeling of soluble protein was determined as before. This was compared with the extent of labeling in cells that were lysed by the above-described procedure before labeling with TNBS. Again, the A_{410} of the crude cytoplasmic protein fraction of control cells that were not trinitrophenylated was subtracted from the readings.

RESULTS

Lipid composition of intact cells and protoplasts of *B. amyloliquefaciens*. The lipid composition of *B. amyloliquefaciens* membranes has not been previously reported, and preliminary experiments were aimed at determining the individual classes of lipid present.

Cells were grown from a spore inoculum to late logarithmic phase and, where required, converted to protoplasts by using lysozyme as described above. The total lipid extract from either cells or protoplasts was fractionated by stepwise elution from a column of silicic acid (Mallinckrodt, 100 mesh) by the method of Bishop et al. (2). Three lipid classes were recognized—neutral lipid, glycolipid, and phospholipid; these comprised, respectively, about 14, 11, and 75% of total lipid by dry weight for either cells or protoplasts. These results are similar to those reported by Bishop et al. (2) for *B. subtilis* 168. Although in the present work no attempt has been made to identify the neutral lipid or glycolipid, it is expected to be similar to that for *B. subtilis*, the neutral lipid fraction consisting of mainly a diglyceride and the glycolipid being a diglucosyl diglyceride (2).

To determine the phospholipid composition, cells were grown from spore inoculum in the presence of ^{32}P-labeled inorganic phosphate to uniformly label phospholipids. The phospholipids were extracted from late-log cells and separated by two-dimensional thin-layer chromatography. The radioactive phospholipid spots were detected by autoradiography and were then scraped off and counted. Alternatively, ^{32}P-labeled cells were converted to protoplasts before lipid extraction. The phospholipid composition of both cells and protoplasts is shown in Table 1. The three major phospholipids are CLP, PG, and PE. It is apparent that when cells are converted to protoplasts, a marked alteration in the relative proportions of CLP and PG occurs. The total loss of ^{32}P counts from PG was found to be about equal to the increase in ^{32}P counts in CLP. Further experiments showed that after the initial 60-min incubation with lysozyme, no further significant alteration in the relative proportions of phospholipid occurred in the protoplast membrane. Furthermore, when lysozyme only was omitted from the protoplast incubation medium, this alteration was not observed. It has been reported by Hirschberg and Kennedy (9) that PG can be directly converted to CLP in *Escherichia coli*, and possibly such a direct conversion occurs during protoplast formation in *B. amyloliquefaciens*. Op den Kamp et al. (12) have reported an apparently similar, but less marked, conversion, in *B. subtilis* Marburg during protoplast formation.

The remaining 9% of phospholipid in both cells and protoplasts consisted of several small components that did not migrate far from the origin on the thin-layer chromatography plates. These minor components were not identified but were assumed to be lipoamino acids since they contained a phosphate group, were ninhydrin positive, and did not comigrate with PE, phosphatidylserine, or lysophosphatidylethanolamine. Lipoamino acids have been found in similar proportions in *B. subtilis* (2, 12).

Treatment of intact protoplasts with phospholipase C and phospholipase A_2. ^{32}P-labeled protoplasts were suspended in protoplast medium and incubated with either phospholi-

TABLE 1. *Phospholipid composition of cells and protoplasts of B. amyloliquefaciens*[a]

Component	Cells	Protoplasts
CLP	9 (1,379)	30 (3,801)
PG	52 (3,949)	29 (1,825)
PE	30 (2,248)	32 (2,053)
Remainder	9 (672)	9 (582)

[a] Phospholipids were extracted from whole cells immediately after lysis in a French pressure cell, or from cells that were converted to protoplasts. The phospholipids were separated and quantitated as described in the text, and the proportions of each phospholipid are expressed as a molar percentage of total phospholipid. The original ^{32}P counts per minute above background are given in parentheses. Since CLP has two phosphate groups per molecule, CLP counts have been halved before calculation of the molar percentages.

pase C (*B. cereus*) or phospholipase A_2 (*Crotalus*). The action of phospholipases on the phospholipids was determined by following the loss in radioactivity in the individual phospholipids isolated by thin-layer chromatography. The time course of hydrolysis of the three major phospholipid species by these enzymes is shown in Fig. 1. In the presence of phospholipase C, about 70% of the total phospholipid in the protoplast membrane is degraded after 1 h at 37°C. About 90% of total PE and PG is degraded compared with only 30% of CLP.

A similar result was obtained when protoplasts were incubated with phospholipase A_2, although the initial rates of degradation were slightly less for PE and PG.

When ^{32}P-labeled protoplasts were incubated for 1 h at 37°C in the absence of added phospholipase and the phospholipids were isolated at different times, there was no alteration in the amount of any of the phospholipid species.

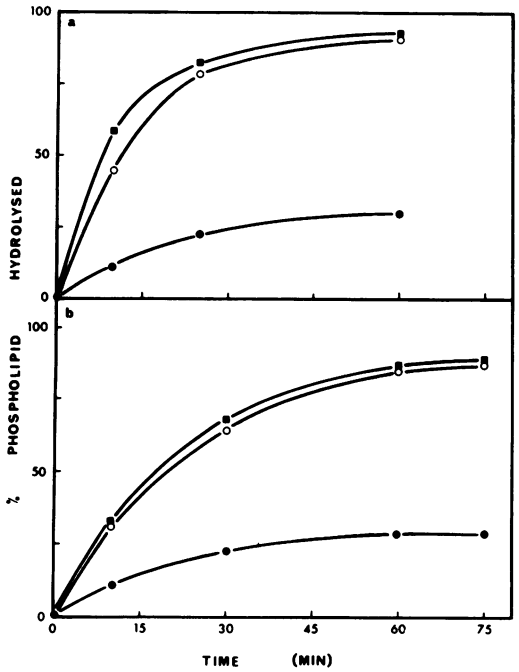

FIG. 1. *Effect of phospholipases A_2 and C on the phospholipids of B. amyloliquefaciens protoplasts. Portions (1 ml) of ^{32}P-labeled B. amyloliquefaciens protoplasts in protoplast medium, each containing about 100 μg of phospholipid, were incubated at 37°C with: (a) 30 IU of phospholipase C (B. cereus); (b) 4 IU of phospholipase A_2 (Crotalus) + 10 μl of 0.1 M $CaCl_2$. At the indicated times, the reaction was stopped by the addition of 0.1 ml of 1 M EDTA, the lipids were extracted, and the phospholipids were quantitated as described in the text. The amount of CLP (●), PG (○), and PE (■) degraded is expressed as a percentage of the total amount of each phospholipid in protoplasts that were incubated for the same time in the absence of added phospholipase.*

Incubation of protoplasts at 37°C with twice the amount of enzyme (A_2 or C) and for longer time periods did not result in any further degradation of any of the phospholipid species.

Treatment of permeablized protoplasts with phospholipases A_2 and C. It has previously been shown in our laboratory (18) that when cells of *B. amyloliquefaciens* are rapidly chilled (cold shocked), by dilution in ice-cold media, there is a breakdown in the permeability barrier of the cells. This breakdown permits small proteins to cross the membrane, although the cells, as judged by electron microscopy, remain intact. Up to 85% of an intracellular ribonuclease inhibitor (molecular weight, 12,000) can be released from cells by this treatment. A similar phenomenon is observed when protoplasts of this organism are rapidly chilled in the presence of sucrose; again, 85% of the intracellular ribonuclease inhibitor is released, although the protoplasts remain intact (J. C. Paton, unpublished data).

To investigate whether additional phospholipid could be degraded in these permeabilized protoplasts, such protoplasts were incubated with either phospholipase C or A_2 for 60 min at 37°C. The phospholipids were extracted, separated, and quantitated as before. The results (Table 2) show that nearly three times more CLP is exposed to phospholipase C in cold-shocked, compared with normal, protoplasts. In addition, PG and PE are further degraded to 98 and 99%, respectively. When phospholipase A_2 is used, again, PG and PE are degraded almost to completion while CLP is digested to 71%.

Effect of phospholipases A_2 and C on the integrity of protoplasts. During incubation with either phospholipase A_2 or C, the protoplasts appeared, by phase-contrast microscopy, to remain intact even after 75 min at 37°C. However, it was important to have a quantitative analysis of the membrane integrity.

The A_{600} of protoplast suspensions was monitored during incubation with each phospholipase and did not decrease over 60 min at 37°C (Fig. 2). However, when protoplasts are resuspended in buffer without sucrose, causing total lysis, the A_{600} decreases to about 5% of the initial value. There is a gradual increase in the A_{600} of the protoplast suspensions whether phospholipases are present or not, which is due to swelling of protoplasts resulting from glutamate uptake from the medium (May, unpublished data).

As a further check upon the impermeability of the protoplast membrane to proteins in the presence of phospholipase C or A_2, the leakage

TABLE 2. *Digestion of phospholipids on intact and permeabilized protoplasts by phospholipases A_2 and C*[a]

Component	Phospholipase C						Phospholipase A_2					
	Intact			Permeabilized			Intact			Permeabilized		
	No enzyme (cpm)	With enzyme (cpm)	Hydrolyzed (%)	No enzyme (cpm)	With enzyme (cpm)	Hydrolyzed (%)	No enzyme (cpm)	With enzyme (cpm)	Hydrolyzed (%)	No enzyme (cpm)	With enzyme (cpm)	Hydrolyzed (%)
CLP	3,924	2,741	30	3,426	659	80	3,856	2,728	29	3,662	1,052	71
PG	1,863	169	91	1,618	29	98	1,790	231	87	1,753	72	96
PE	2,040	139	93	1,884	22	99	2,002	218	89	2,021	15	99

[a] Portions (1 ml) of ^{32}P-labeled intact protoplasts or protoplasts permeabilized by cold-shock treatment (each containing about 100 μg of phospholipid) were incubated in protoplast medium with or without either 30 IU of phospholipase C, or 4 IU of phospholipase A_2 with 10 μl of 0.1 M $CaCl_2$ for 60 min at 37°C. The reaction was then stopped by the addition of 0.1 ml of 1 M EDTA, and the phospholipids were extracted, separated, and quantitated as described in the text. Counts per minute above background in the presence or absence of enzyme are shown. The percent hydrolysis of each phospholipid species is also given.

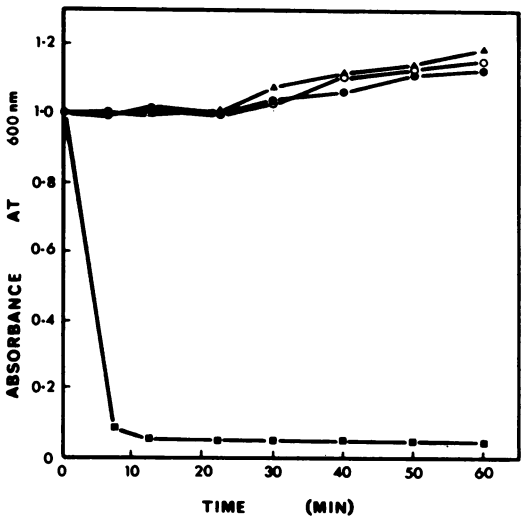

FIG. 2. *Effect of phospholipases A_2 and C on A_{600} of protoplast suspensions. Suspensions of protoplasts in protoplast medium containing about 100 μg of phospholipid per ml were incubated at 37°C in the presence of 4 IU of phospholipase A_2 (Crotalus) per ml* (▲), *30 IU of phospholipase C (B. cereus) per ml* (○), *or with no added enzyme* (●). *Samples were withdrawn at intervals, and A_{600} was measured in a Hitachi model 101 spectrophotometer. As a comparison, the A_{600} of protoplasts suspended in buffer without sucrose was followed* (■), *total lysis having occurred within 10 min.*

of the intracellular ribonuclease inhibitor (18) was examined over a 60-min incubation. This inhibitor binds irreversibly with the ribonuclease secreted by *B. amyloliquefaciens* when the two are mixed together. Protoplasts were incubated for 60 min at 37°C with or without either phospholipase and centrifuged at 4,000 × *g* for 5 min, and the amount of ribonuclease inhibitor present in the supernatant fraction was assayed as described above. The total amount of inhibitor present in the protoplasts was determined by lysing them in a French pressure cell at 15,000 lb/in^2, centrifuging as before, and assaying the supernatant fraction for ribonuclease inhibitor. It was found that about 4% of the total intracellular inhibitor was released from intact protoplasts in the presence or absence of either phospholipase. It is therefore clear that the phospholipases do not promote either protoplast lysis or significant alteration in the permeability properties of the protoplast that might permit free passage of proteins across the membrane during the period of incubation.

Phospholipase C digestion of intact cells. Phospholipase digestion of the membrane phospholipids in protoplasts suggested that about 90% of PE was externally located in the bilayer, and, to confirm this, the chemical reagent TNBS was employed to modify external PE. Experiments with TNBS were performed with intact cells since the conditions required for TNBS modification were not suitable for protoplasts of *B. amyloliquefaciens*. The unlikely possibility that the distribution of PE, as judged by phospholipase treatment, in intact cells differs from that in protoplasts was checked by incubating intact cells with phospholipase C. Although the rates of degradation of PE, PG, and CLP in intact cells are considerably slower than that seen with protoplasts under identical conditions, and in fact had not gone to completion in 90 min (Fig. 3), the patterns of degradation of the three phospholipids are similar to those of protoplasts. At least 80% of PE in intact cells is accessible to the enzyme.

Extent of penetration of TNBS into cells. If TNBS was to be used to label externally localized PE, it was important to establish conditions in which it did not penetrate the cell membrane. To examine this, cells were incubated with 3 mM TNBS at 37°C for 30 min, and the extent of trinitrophenylation of cytoplasmic protein was determined by A_{410} as described

above. This value was compared with the degree of labeling of an isolated cytoplasmic protein fraction prepared from cells by treatment in the French pressure cell.

From Table 3 it is clear that at 37°C cells are readily permeable to TNBS since greater than 60% of cellular cytoplasmic protein was modified. However, when the modification was carried out at 4°C (for 4 h) the entry of TNBS into the cells was considerably less (Table 3). The fraction of cytoplasmic protein isolated from the labeled cells had about 20% of the color due to trinitrophenyl groups found in a previously isolated cytoplasmic protein fraction that was incubated with TNBS. (This result is quantitatively similar to that found by Rothman and Kennedy [13] when *B. megaterium* cells were treated with TNBS at 3°C, and these authors suggested that at 37°C a membrane transport protein was responsible for the rapid entry of TNBS.)

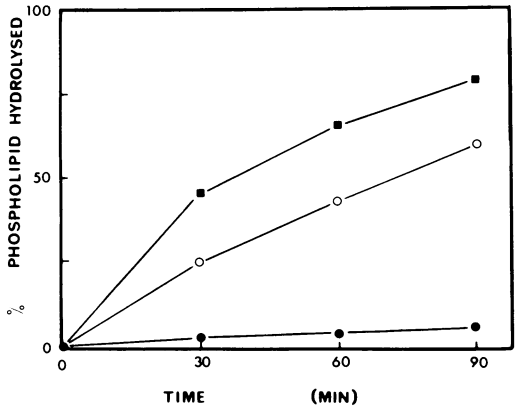

FIG. 3. *Effect of phospholipase C on the phospholipids of intact cells of B. amyloliquefaciens. Portions (1 ml) of cells, suspended in 50 mM Tris-hydrochloride, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ (pH 7.3), and containing about 100 μg of phospholipid were incubated with 30 IU of phospholipase C (B. cereus) at 37°C. The percentage of the total amount of CLP (●), PG (○), and PE (■) degraded was determined as described in the legend to Fig. 1.*

TABLE 3. *Extent of penetration of TNBS into cells*[a]

Lysis procedure	Incubation conditions	A_{410} of crude cytoplasmic protein fraction		A_{410} of cytoplasmic protein of intact cells as a percentage of A_{410} of the isolated protein fraction labeled under the same conditions
		Intact cells	Isolated protein fraction	
French pressure cell lysis	37°C, 30 min	0.708	1.108	63.9
	4°C, 4 h	0.227	1.017	22.3
Osmotic lysis	4°C, 4 h	0.025	0.850	2.9

[a] Cells were incubated with 3 mM TNBS under various conditions, washed, and then lysed either by French pressure cell treatment or by conversion to protoplasts followed by osmotic lysis. The lysates were then centrifuged (105,000 × *g*, 60 min), and the A_{410} of supernatant protein was determined as described in the text. This value was compared with that of a previously isolated cytoplasmic protein fraction that was labeled with TNBS under the same conditions. The A_{410} of the cytoplasmic protein fraction of cells not labeled with TNBS was 0.010 and was subtracted from all readings to yield the above values.

However, even at 4°C there does appear to be some labeling of cytoplasmic protein in intact cells treated with TNBS. This apparent labeling may be due to labeling of extracytoplasmic proteins which would be released by French pressure cell treatment and would not be separated from cytoplasmic protein by the method employed. To investigate this possibility, instead of lysing cells in the French pressure cell after labeling, the cells were thoroughly washed and converted to protoplasts. The protoplasts were washed with protoplast medium and lysed osmotically by dilution into buffer lacking sucrose; phase contrast microscopy showed that no intact protoplasts remained. The lysate was centrifuged, and the degree of labeling of supernatant (cytoplasmic) proteins was determined as before and compared with the extent of labeling of a cytoplasmic protein fraction isolated by this latter procedure and then trinitrophenylated. Less than 3% of cytoplasmic protein was labeled by TNBS in intact cells (Table 3), suggesting that at 4°C there is very little, if any, penetration of TNBS into the cells.

Modification of PE by TNBS. Cells uniformly labeled with ^{32}P were incubated with 3 mM TNBS at 4°C. Samples were withdrawn at intervals, the reaction was stopped, and the phospholipids were extracted, separated, and quantitated. The chemical modification was followed by the loss of radioactivity from the PE spot and its appearance in the trinitrophenyl-PE spot. The reaction was virtually complete after 3 h, and a maximum of 92% of the total cellular PE was trinitrophenylated (Fig. 4).

To ensure that there was sufficient TNBS to react with all available PE, ^{32}P-labeled cells were incubated with various concentrations of TNBS for 4 h at 4°C. The extent of PE labeling increased with TNBS concentration until a saturation point was reached at about 2 mM TNBS (Fig. 5). The maximum amount of PE that could be modified, even in the presence of 6 mM TNBS, was 93%.

DISCUSSION

The major phospholipids extracted from *B.*

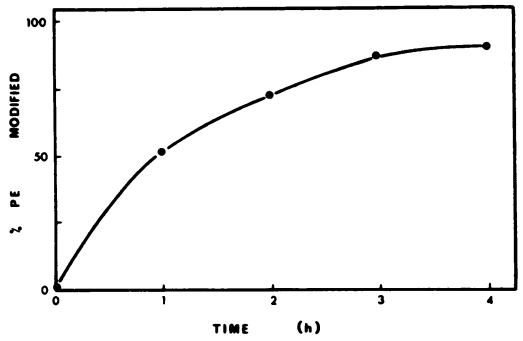

FIG. 4. *Modification of PE by TNBS. ^{32}P-labeled cells were incubated with 3 mM TNBS at 4°C. Samples were withdrawn at the indicated times, the reaction was stopped, and the percentage of the total amount of PE that had been trinitrophenylated was determined as described in the text.*

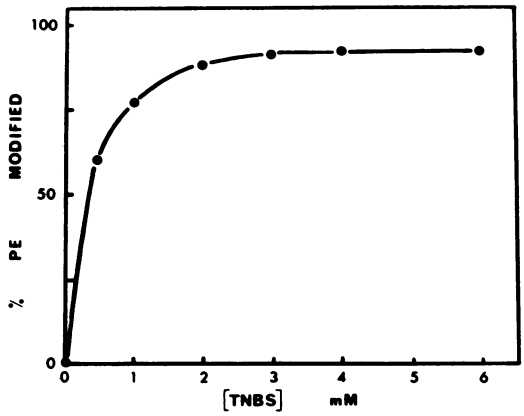

FIG. 5. *Modification of PE by TNBS at different concentrations. Portions of ^{32}P-labeled cells were incubated with different amounts of TNBS at 4°C. After 4 h, the reaction was stopped and the percentage of the total amount of PE that had been modified was determined as described in the text.*

amyloliquefaciens are CLP, PG, and PE. Since the mesosomal membrane represents only a small proportion of the total and has the same composition as the cytoplasmic membrane (8), it may be assumed that these phospholipids are the predominant components of the cytoplasmic membrane. Of the total phospholipid found in *B. amyloliquefaciens* cells, 9% is CLP, 52% is PG, and 30% is PE. During protoplast formation, the PG:CLP ratio decreases to a constant value such that CLP, PG, and PE represent 30, 29, and 32%, respectively, of the total phospholipid found in the protoplast cytoplasmic membrane. The apparent conversion of PG to CLP observed here also occurs during protoplast formation in *B. subtilis* Marburg (12). Such a conversion may imply that a transmembrane rearrangement of phospholipid occurs during protoplast formation.

Studies on the hydrolysis of phospholipids of intact protoplasts have been carried out, both with phospholipases A_2 and C; incubation of protoplasts at 37°C with either of these resulted in 70% of the total phospholipid being degraded, indicating an asymmetric distribution of total phospholipid.

The membrane of this organism consists of 60% protein (by weight), and electron micrographs of freeze-fractured protoplasts have revealed that the protein particle arrangement in the bilayer is highly asymmetric. From the average diameter and particle density, it has been calculated that, by volume, about 75% of membrane protein is associated with the inner monolayer (unpublished data). This necessitates a highly asymmetric distribution of membrane lipid, and the observed phospholipid distribution is not unexpected.

Phospholipase treatment of protoplasts degraded 90% of the PE and PG, but only 30% of the CLP. Under the conditions used, the protoplasts remained intact. No decrease in A_{600} occurred during incubation with the enzymes, and there was no release of intracellular ribonuclease inhibitor. The latter is a protein of molecular weight 12,000 which is known to readily diffuse from cells which have been cold shocked (18). Since this protein is of smaller molecular weight than the phospholipases, it seems unlikely that the latter could have penetrated the protoplasts. This conclusion is confirmed by the fact that when protoplasts were rendered permeable to large molecules by cold shock treatment, the digestion by phospholipases increased to 98, 99, and 80% for PG, PE, and CLP, respectively. Incomplete hydrolysis of CLP may be due to a small fraction of protoplasts which have escaped cold shocking (only 85% of the total intracellular inhibitor is released). Rapid resealing of some protoplasts after cold shocking or shielding of phospholipids by proteins are alternative explanations. Experiments, with liposomes prepared by sonification of total extracted phospholipid, showed that a 90% degradation of CLP occurred with phospholipase C and essentially all PG and PE were digested (Paton, unpublished data). The reason for the 10% of CLP remaining undigested is not clear.

The results with the phospholipases suggested a marked asymmetry in the distribution of both total phospholipid and individual phospholipid species. An asymmetrical distribution of PE with about 90% present in the outer monolayer was

confirmed by experiments with TNBS, which labeled 92% of cellular PE in intact cells. This conclusion depends on the assumption that TNBS does not penetrate the cell membrane. The procedure to check this used by Rothman and Kennedy (13) was to compare the conjugation of TNBS with cytoplasmic protein of intact cells with that occurring in toluenized cells. The value they obtained (17%) with *B. megaterium* was close to the value (22%) obtained with *B. amyloliquefaciens* labeled before or after lysis in a French pressure cell. This value is disturbingly high, since it might imply considerable penetration by the reagent. However, "cytoplasmic" protein fractions prepared by this method would also contain peripheral proteins, membrane protein shed from the bilayer during French pressure cell treatment, and possibly minute fragments of cell wall material, which could give an erroneously high value, as was pointed out, but not tested by Rothman and Kennedy (13). When similar penetration experiments were carried out on *B. amyloliquefaciens*, using a lysis technique that did not permit such contamination of the cytoplasmic protein fractions, a value of only 3% labeling of cytoplasmic protein was obtained. This value indicates that there is little or no penetration of cells by TNBS.

The TNBS labeling experiments were performed at 4°C, a temperature at which the cytoplasmic membrane of this organism is in the gel phase as indicated by differential scanning calorimetry (Paton, unpublished data). This eliminates the unlikely possibility that PE flip-flop occurs during modification with TNBS. Since the amount of external PE labeled by TNBS is identical to that amount hydrolyzed by phospholipases in intact protoplasts, it is apparent that PE flip-flop does not occur during phospholipase treatment. The digestion by phospholipases of CLP and PE suggests that these phospholipids are also arranged asymmetrically in the bilayer with 30% CLP and 90% PE being external. However, the possibility cannot be eliminated that flip-flop of these phospholipids occurs during enzymatic treatment. This seems unlikely, since the results of Barsukov et al. (1) indicate that phospholipase-induced flip-flop of these particular phospholipids did not occur in *M. lysodeikticus*.

Since the completion of this work, Bishop et al. (3) reported somewhat similar results to those reported in this work for PE in *B. subtilis* protoplasts. Digestion of 90% of PE occurred with phospholipase C in protoplasts, but only 60% of PE could be labeled with TNBS. To account for this discrepancy, these authors suggested that 30% of the "external" PE was the result of flip-flop caused by phospholipase treatment. No direct evidence for this proposal was presented. However, the overall conclusion is that at least 60% of PE in the *B. subtilis* membrane is in the outer monolayer.

According to Rothman and Lenard (14), during membrane biogenesis, individual phospholipids are synthesized on the cytoplasmic side of the bilayer and some of this lipid is translocated to the outer monolayer. The distribution of phospholipids would presumably reflect the relative rates of synthesis and "translocation" for each phospholipid species. Unless the rate of translocation was precisely half that of synthesis, an asymmetric membrane would always result.

It is possible that the relative rates of synthesis and translocation of a given phospholipid species are deliberately controlled to achieve a particular asymmetry. It is also possible, however, that the rates are essentially fortuitous and the asymmetry is equally without fundamental significance. This latter proposal is supported by the present finding that the PE distribution in the cytoplasmic membrane of *B. amyloliquefaciens* differs markedly from that found in *B. megaterium* (13), indicating that there is no overriding need for a particular orientation of PE in bacterial membranes.

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