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Action of Phospholipase A_2 and Phospholipase C on *Escherichia Coli*

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The action of exogenous phospholipases on *Escherichia coli* has been examined. Cells harvested in late log phase were found to be completely resistant to the action of phospholipases A_2 and C. Treatment of cells with Tris and EDTA was required to make the phospholipids in the cell accessible to these phospholipases. Phospholipase A_2 hydrolyzed mainly phosphatidylethanolamine and phosphatidylglycerol, whereas phospholipase C preferentially degraded phosphatidylethanolamine.

During the EDTA treatment, an endogenous phospholipase A_1 or a lysophospholipase (or both) was unmasked which caused the formation of free fatty acids in experiments in which no phospholipase was added and which degraded some of the lysophospholipids formed by phospholipase A_2.

The cells were rapidly killed by the successive Tris-EDTA-phospholipase treatment, but no cell disintegration was observed.

The cell envelope of *E. coli* is composed of three distinct layers: an inner cytoplasmic membrane, a murein or peptidoglycan layer which presumably gives the cell its characteristic shape, and an outer or L membrane (1-4). Historically, the inner membrane has been referred to as the cell membrane while the peptidoglycan and outer membranes were referred to (collectively) as wall. The inner membrane is very similar to other biological membranes in that it contains the proteins of the terminal respiratory chain and acts as a permeability barrier for the cell. In addition, enzymes of phospholipid synthesis are found associated with the inner membrane (5, 6). The peptidoglycan layer of gram-negative bacteria is analogous to the cell wall of gram-positive organisms where it is a much thicker structure and the outermost layer of the cell. In *E. coli* and other gram-negative organisms, the rigid peptidoglycan layer is surrounded by another membranous layer which, in addition to phospholipid and protein arranged in a characteristic "railroad track" membrane, contains most of the lipopolysaccharide of the cell envelope. The phospholipase A_1 of *E. coli* has been shown to be localized in the outer membrane (7).

The outer membrane contributes to a barrier within the cell wall which excludes a wide variety of molecules and also retains enzymes and other components within the periplasmic space. Treatment with EDTA disrupts the barrier function of the outer membrane and also causes the release of a lipopolysaccharide-lipid-protein complex (8-11). One consequence of the changes brought about by EDTA treatment is that the peptidoglycan is made accessible to exogenously added lysozyme (10).

Studies in other laboratories have indicated that the outer membrane must be modified before the phospholipids of intact *E. coli* can be hydrolyzed by exogenous phospholipases. Slein and Logan (12) observed that *E. coli* is lysed in the presence of phospholipase C only after treatment of the cells with EDTA. Weinbaum *et al.* (13)

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showed that phospholipases A, C, and D are not able to alter the morphology of *E. coli* but that a combination of protein hydrolysis with trypsin and phospholipase C or D action converts rod shaped cells into spheres. These observations cannot be accurately interpreted, however, as commercially available phospholipase C preparations have been found to contain an endo-*N*-acetyl-glucosaminidase activity which may have been responsible for the degradation of the murein, resulting in lysis or sphere formation (14). The availability of highly purified phospholipase A_2 and C preparations has made it possible to reinvestigate the effects of these enzymes on *E. coli*. We observed that membrane phospholipids of intact cells are hydrolyzed only if the cells are pretreated with EDTA, but cell lysis does not occur even after extensive hydrolysis of the membranes with either enzyme. Sphere formation was observed but could not be directly correlated with phospholipase C action because the EDTA treatment itself was followed by sphere formation.

EXPERIMENTAL PROCEDURES

Preparation of Cells

Escherichia coli B, a wild-type strain obtained from the laboratory of M. J. Bessman, was cultivated at 37°C in an alkaline broth medium (15). Late log-phase cultures were obtained by inoculating 125 ml of the same medium with 1.5 ml of an overnight culture and shaking for 3 hr at 37°C. This resulted in an absorbance of approximately 1.2 at 550 nm (Unicam Sp 500 Spectrofotometer, 1-cm light path). The cells were harvested by centrifugation and washed once with 0.12 M Tris buffer, pH 7.8, containing 5×10^{-4} M $MgCl_2$. After washing, the cells were suspended in 0.12 M Tris buffer, pH 7.8, without the magnesium, at a concentration of about 2.5×10^{10} cells per ml.

Radioactive Labeling of Cells

Routinely, the phospholipids were labeled by the addition of 200 μCi of $H_3{}^{32}PO_4$ to 125 ml medium. Where indicated, [^{14}C]oleic acid or [^{14}C]palmitic acid was used to label the lipids. [1-^{14}C]Oleic acid with a specific activity of 0.8 μCi/mmole was added in a small amount of ethanol (0.4 μCi per 125 ml medium). Four-tenths microcurie [1-^{14}C]palmitic acid was mixed with 0.5 μmoles of oleic acid and added to 125 ml medium which contained 40 mg fatty acid-poor bovine serum albumin (fraction V powder, Calbiochem). When labeling of the ribonucleic acids was required, 2.5 μCi of [2-^{14}C]uracil (62 Ci/mole) was added per 125 ml of medium.

Cell Treatment

The routine starting material in most experiments consisted of cells treated briefly with EDTA followed by the addition of excess $CaCl_2$. The addition of the Ca^{2+} was necessary for the action of the phospholipases. The protocol for this treatment is as follows: To 1 ml of cells in 0.12 M Tris-HCl buffer, pH 7.8, EDTA was added to a final concentration of 5×10^{-4} M. After 2 min, the action of EDTA was stopped by the addition of 50 μl of 0.1 M $CaCl_2$ per ml of cell suspension.

Phospholipase A_2 treatment was carried out with purified porcine pancreatic phospholipase A_2 (EC 3.1.1.4) generously contributed by Dr. G. H. de Haas. It was dissolved in water at a concentration of 1 unit per μliter. The purified enzyme will remove only the fatty acid at the 2-position of phospholipids (16). Routinely, 1 ml of cell suspension treated with EDTA and Ca^{2+} as above was incubated with 10 μl of phospholipase A_2 solution at 37°C. The reaction was stopped by the addition of 0.2 ml saturated EDTA solution.

For treatment with phospholipase C (EC 3.1.4.3), the purified enzyme from *Bacillus cereus* was used (17). The enzyme was dissolved in 0.01 M Tris buffer, pH 7.4, containing 50% glycerol at a concentration of 0.5 units per μliter (17). Incubations were carried out with 1 ml of Tris-EDTA-Ca^{2+}-treated cells containing 10 μl of this enzyme preparation. The reaction was stopped by adding 0.1 ml of 0.02 M phenanthrolinium chloride.

Lipid Analysis

The incubation mixture was cooled in ice and 1.5 ml 10% trichloroacetic acid was added. Lipids were extracted with chloroform and methanol followed by washing of the chloroform layer with 1 M citric acid and 1 M acetic acid. In some experiments, the phospholipids were extracted in the absence of trichloroacetic acid, but this did not allow recovery of the free fatty acids. Control experiments showed that by extraction in the presence of trichloroacetic acid, maximal recovery of lysophospholipids and free fatty acids was obtained. The lipids were chromatographed on Silica-gel H thin-layer plates, developed in chloroform–methanol–water–acetic acid (65:25:4:1) at 4°. The lipids were detected with iodine vapor and identified by their differential staining with ninhydrin and phosphate stain, and also by comparison with authentic reference compounds. This solvent system gave excellent separation of all componenets. Characteristic R_F values were as follows: lysophospholipids, 0.15–0.25; phosphatidyl glycerol, 0.45; phosphatidyl ethanolamine, 0.6; cardiolipin, 0.9. Free fatty acids were found at the solvent front. Phosphatidyl choline,

when added as an internal standard, was found between the lysocompounds and phosphatidyl glycerol. The silica gel was scraped off the plate and counted in a mixture of toluene (containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis[2-(5-phenyloxazolyl)]-benzene), Triton X-100, and water (2:1:0.2) in a Packard scintillation counter. In some experiments corrections for losses during extraction were made by calculating the recovery of a known amount of [^{14}C]phosphatidylcholine added to the cells after incubation.

Permeability Measurements

β-Galactosidase leakage was determined with cells in which the synthesis of this enzyme was induced by the addition of 5×10^{-4} M isopropyl-β-D-thiogalactopyranoside to the growth medium. During the incubation of EDTA-treated cells with or without phospholipases, samples were taken and rapidly centrifuged at 22° in a Beckman microfuge. Part of the supernatant was removed and assayed for the presence of β-galactosidase as described by Slein and Logan (12). The sonication procedure described by these authors was used to determine the total β-galactosidase content of the cells.

RNA leakage was measured using [^{14}C]uracil-labeled cells and the same centrifugation technique as described for the β-galactosidase assay. When indicated, the RNA was measured after being precipitated with 5% trichloracetic acid and collected by filtration.

Viability Measurements

To test for viability after the different washing and incubation procedures, cells were serially diluted into alkaline broth medium (15) and aliquots plated by the agar-layer technique onto plates containing alkaline broth agar.

Electron Microscopy

Samples taken directly from the different incubation mixtures were fixed by the addition of OsO_4 at a final concentration of 1%. Samples were dehydrated with acetone and embedded in araldite. Ultrathin sections were stained with saturated uranyl nitrate and 1% lead citrate. Electron micrographs were made with a Siemens Elmiskop 1A.

RESULTS

Phospholipid Content of Intact E. coli B

Numerous determinations with cells grown in alkaline broth medium and labeled with $H_3{}^{32}PO_4$ have shown the phospholipids of *E. coli* B to be composed of the following: lysophospholipids, 1–3%; phosphatidyl glycerol, 15–19%; phosphatidyl ethanolamine, 72–78%; cardiolipin, 5–7%. Analyses of cells grown with [^{14}C]palmitic acid or [^{14}C]oleic acid indicate the following composition: lysophospholipids, 1–2%; phosphatidyl glycerol, 17–18%; phosphatidyl ethanolamine, 72–78%; cardiolipin, 4–8%; free fatty acids, 1–2%. (The numbers given indicate the range of values obtained.)

Degradation of Phospholipid

When intact cells of *E. coli* B were incubated with phospholipase A_2 or C, no degradation of the phospholipids occurred (Fig. 1). Treatment of the cells with Tris and EDTA allowed phospholipid breakdown to occur in the presence of phospholipases provided that an excess of $CaCl_2$ was added after incubation with Tris-EDTA. When Tris-EDTA-treated cells were incubated at 37°C with phospholipase A_2 and $CaCl_2$, a rapid hydrolysis of the phospholipids took place whereas after the addition of comparable amounts of phospholipase C hydrolysis proceeded at a lower rate. The observation that phospholipase A_2 and, at higher enzyme concentrations, phospholipase C were able to degrade more than 90%

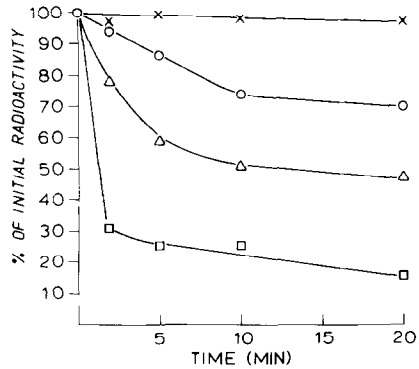

FIG. 1. Effect of phospholipases on the total phospholipid content of *E. coli*. Cells were grown in the presence of $H_3{}^{32}PO_4$ to label the phospholipids. After harvesting at an OD of 1.2 at 550 nm the cells were incubated under conditions described below. At different times, the remaining phospholipids (including lysocompounds) were isolated and their radioactivity counted. (X—X) Incubation in Tris + phospholipases A_2 or C in the presence of $CaCl_2$ and incubation of Tris-EDTA-treated cells in the absence of $CaCl_2$. (O—O) Tris-EDTA-treated cells, incubated with $CaCl_2$. (Δ—Δ) Tris-EDTA-treated cells, incubated with phospholipase C and $CaCl_2$. (□—□) Tris-EDTA-treated cells, incubated with phospholipase A_2 and $CaCl_2$.

of the phospholipids indicated that both enzymes could penetrate the outermost barrier of the Tris–EDTA-treated cells and hydrolyze both the outer membrane and the cytoplasmic membrane phospholipids.

Figure 1 also shows that an endogeneous lipolytic activity is unmasked by the Tris–EDTA treatment. When the Tris–EDTA treatment is not followed by addition of exogenous phospholipases, hydrolysis of as much as 30% of the membrane phospholipid can nonetheless occur. This hydrolysis occurs at a rather slow rate and results indicate that both a phospholipase A_1 activity and a lysophospholipase which are present in *E. coli* (6, 7, 18, 19) are responsible. One of these endogenous lipolytic activities is thought to be the cause of the degradation of lysophospholipase A_2 and $CaCl_2$. After degradation of phospholipids in ^{32}P-labeled cells by phospholipase A_2, only a small portion of the ^{32}P was recovered in the lysophospholipid fraction. The loss of most of the label was not due to an incomplete extraction of lysophospholipids as is demonstrated with the data obtained with sonicated lipid extracts or with cells in which the phospholipids were labeled in the fatty acid moiety (Table I). It was thought that the loss of ^{32}P label might be due to either an endogenous lysophospholipase or a phospholipase A_1 which would cause the labeled product (glycerol phosphate) to be water soluble.

This loss of lysophospholipid was studied using cells labeled with either [^{14}C]oleic acid or [^{14}C]palmitic acid. [^{14}C]Oleic acid-grown cells preferentially incorporated the label at the 2-position of the phospholipid, as can be seen from the data in Table I on the action of highly purified phospholipase A_2 on sonicated isolated lipid extracts. [^{14}C]Palmitic acid was present mainly at the 1-position, although 20% is found in the 2-position. After phospholipase A_2 action on [^{14}C]oleic acid-grown cells, all the label was recovered in the form of free fatty acid, as could be expected. In the case of [^{14}C]palmitate-labeled cells, the radioactivity was also recovered mainly in the free fatty acid fraction, demonstrating the degradation of lysophospholipid into free fatty acid and water-soluble components by an endogenous lipolytic acitivity. The endogenous phospholipase A_1 which is known to hydrolyze 1-acyl lysophospholipids (7) could account for this.

The data presented in Fig. 2 show that phosphatidylethanolamine and phosphatidylglycerol are rapidly hydrolyzed by phospholipase A_2. Part of the phosphatidylglycerol may also be converted into cardiolipin (20) accounting for the initial increase which is observed in the amount of this lipid.

The amount of lysophospholipid which is formed (Fig. 2A) is much smaller than can be expected from the loss of phosphatidylethanolamine and phosphatidylglycerol. Using [^{14}C]palmitic acid, 80% of which is incorporated in the 2-position, shows that the lysophospholipids are degraded further

TABLE I

EFFECTS OF PHOSPHOLIPASE A_2 ON DIFFERENTLY LABELED PHOSPHOLIPIDS OF *E. coli*

Label	Recovery of label from PE and PG in extractable lipid after phospholipase A_2 action[a] on:					
	Cells			Sonicated lipid extracts		
	Total recovery[b]	% Recovery in		Total recovery[b]	% Recovery in	
		Lyso	FFA		Lyso	FFA
^{32}P	10–30	10–30	—	95	95	—
[^{14}C]Oleic acid	100	2	98	100	5	95
[^{14}C]Palmitic acid	100	5–30	70–95	100	80	20

[a] Conditions were used such that greater than 90% of the PE and PG were hydrolyzed.

[b] Data are expressed in % recovery of the initial radioactivity. Abbreviations used are: lysophospholipid, lyso; free fatty acid, FFA; phosphoatidylethanolamine, PE; phosphatidylglycerol, PG.

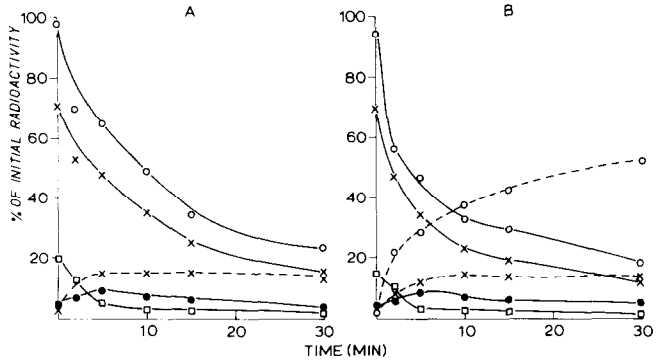

FIG. 2. Effect of phospholipase A_2 on the phospholipids of *E. coli*. Cells were grown in the presence of $H_3{}^{32}PO_4$ (Fig. 2A) and [^{14}C]palmitic acid (Fig. 2B). They were harvested, washed with Tris/Mg^{2++} buffer, and resuspended in Tris. After 2-min incubation with 5×10^{-4} M EDTA, $CaCl_2$ and phospholipase A_2 were added and the incubation was continued at 37°C for the times indicated. The amounts of total phospholipid, (O—O) phosphatidylethanolamine (X—X) phosphatidylglycerol, (□—□), cardiolipin, (●—●), free fatty acids, (---O---), and lysophospholipids, (---X---), are expressed in percentages of the total initial radioactivity.

and that free fatty acids are released (Fig. 2B).

Phospholipase C preferentially hydrolyzed phosphatidylethanolamine in Tris–EDTA-treated cells (Fig. 3). In this case also, the amount of diglyceride formed does not correlate fully with the amount of phosphatidylethanolamine hydrolysed. An accumulation of free fatty acid is also observed. Again, it is thought that this is due to endogenous lipolytic activity which is unmasked by the Tris–EDTA treatment. The nature of this activity is now under investigation.

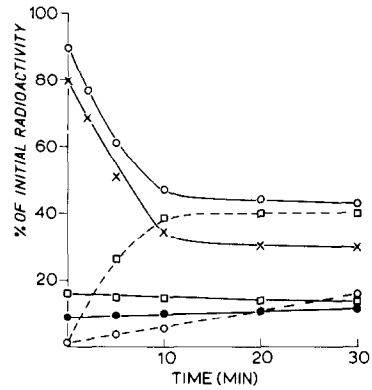

FIG. 3. Effect of phospholipase C on the phospholipids of *E. coli*. [^{14}C]Palmitic acid-grown cells were isolated and incubated exactly as described in the legend to Fig. 2. Phospholipase C was used instead of phospholipase A_2. In this experiment the amount of diglyceride was also determined. Symbols: total phospholipid (O—O), phosphatidylethanolamine (×—×), phosphatidylglycerol, (□—□), cardiolipin, (●—●), free fatty acids (---O---), diglycerides (---□---). The data are expressed as percentages of the total initial radioactivity.

Permeability Effects

The observation that exogenous phospholipases can hydrolyze much more than 50% of the total phospholipids of the cell suggests that these enzymes can penetrate the wall and reach the cytoplasmic membrane. As a consequence of the hydrolysis of the phospholipids in the cytoplasmic membrane, one might expect lysis. We, therefore, studied the release of small- and large-molecular-weight material from treated cells. Table II shows that the amount of β-galactosidase which is released from untreated cells is very small. Successive treatment of the cells with EDTA, Ca^{2+}, and phospholipases does not stimulate the loss of this enzyme except for a very limited increase seen in the case of phospholipase C treatment. Similar results were obtained when the leakage of RNA was studied using [^{14}C]uracil-labeled cells (Table II). Under all conditions, uracil leaked from the cells but was mainly in the form of low-molecular-weight material as judged by its solubility in 5% trichloroacetic acid. Trichloroacetic acid-insoluble material was not released from control cells or

TABLE II

EFFECTS OF PHOSPHOLIPASES ON THE PERMEABILITY AND VIABILITY OF *E. coli*

Treatment	β-galactosidase release[a]	[^{14}C]Uracil release[b]			Viability[c]
		Total	5% TCA insoluble	5% TCA soluble	
None	0.4	10.9	1.1	9.8	100
Tris incubation	0.3				95
Tris-EDTA/Ca^{2+}	0.3	13.0	1.3	11.7	45
Tris-EDTA/Ca^{2+} + phospholipase A_2	0.4	14.0	3.0	11.0	1.4
Tris-EDTA/Ca^{2+} + phospholipase C	0.8	22.0	7.0	15.0	9

[a] Data expressed as percentage of the total amount of β-galactosidase.

[b] Data expressed as percentage of total radioactivity in untreated cells.

[c] Data expressed as percentage of initial number of viable cells. All measurements were carried out after a 20-min incubation period at 37°C. Inhibitors (saturated EDTA or phenanthrolinium chloride) were not added.

EDTA-calcium-treated cells. A relatively small amount of trichloroacetic acid-insoluble material was released after phospholipase A_2 and phospholipase C treatment. This might be due to lysis of a small number of the cells or to a small increase in permeability within each cell. If RNAase was added to cells treated with phospholipase C, an increased leakage of low-molecular-weight [^{14}C]uracil-containing material was obtained (data not included), indicating that the RNAase may have entered the cytoplasm. This was not found after the other treatments.

Electron Microscopy

The permeability studies suggested that the cells do not disintegrate even after extensive phospholipid hydrolysis. This was confirmed by electron microscopy. When *E. coli* cells are treated with Tris-EDTA and subsequently incubated at 37°C with $CaCl_2$ alone (Fig. 4), $CaCl_2$ and phospholipase A_2 (Fig. 5), and $CaCl_2$ and phospholipase C (Fig. 6), the cytoplasmic material appears to be retained within the cell. The ultrastructure of the membranes as visualized by the positive-straining technique also seems to have been preserved. As mentioned in the introduction, a high percentage of spheres is observed when Tris-EDTA cells are treated with phospholipase C (Fig. 6). However, Tris-EDTA treatment followed by an incubation in the presence of Ca^{2+} alone can also result in sphere formation (Fig. 4). It cannot be concluded, therefore, that this transition is phospholipase C dependent.

Viability

Despite the apparent intactness of the cells at all stages of treatment, the viability is drastically reduced by the phospholipase treatments (Table II). In cells treated only with Tris and EDTA, a 25% breakdown of phospholipids is correleated with the death of 55% of the cells. With shorter incubation times where the phospholipid breakdown is still linear with time, the correlation is even more dramatic, indicating that a relatively small amount of phospholipid breakdown may be able to cause cell death. With phospholipase C, breakdown of 50% of the phospholipids results in death of more than 90% of the cells. With phospholipase A_2, viability was almost completely eliminated even when measured within 2 min of the addition of enzyme.

DISCUSSION

Treatment of *E. coli* with EDTA in the presence of Tris has many effects on the cell which have been previously well documented (4, 8–11, 22). To summarize, this treatment causes loss of enzymes of the periplasmic space, increases the permeability of the cell for some small molecules, allows hydrolysis of the murein layer by lysozyme, and releases a complex lipopolysaccharide. Data presented here show that treatment of the cells with EDTA followed by the addition of $CaCl_2$ also allows the

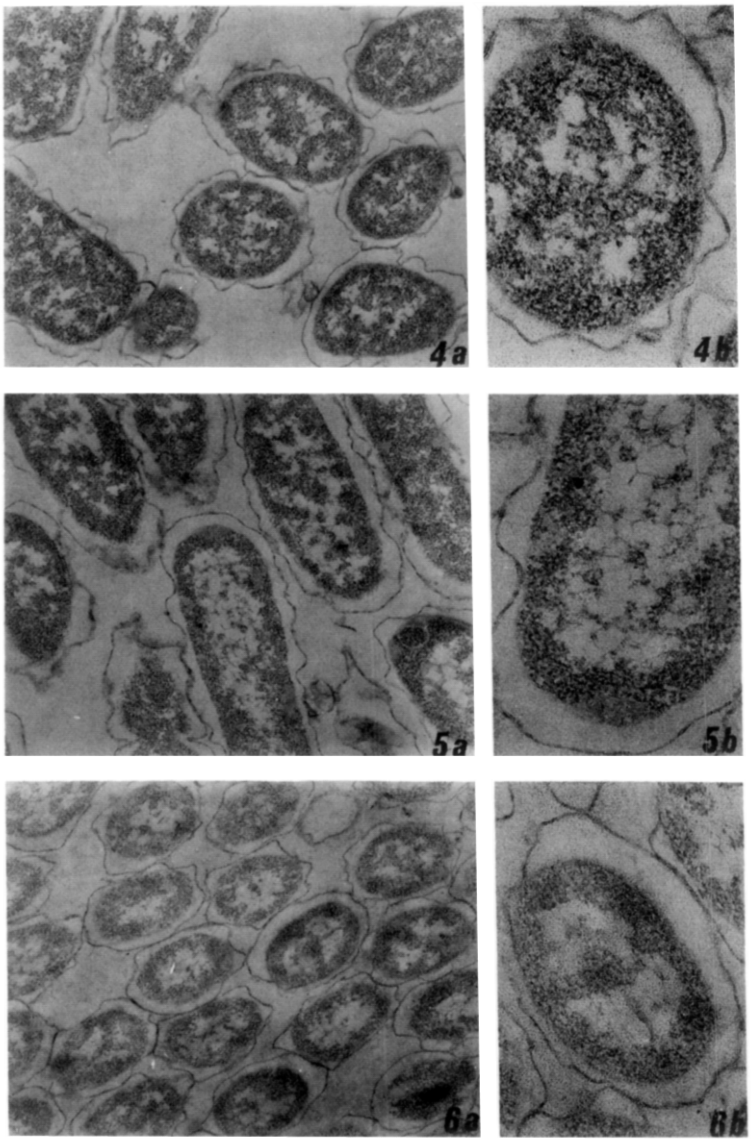

FIG. 4. Thin-sectioned *E. coli* cells treated with Tris-EDTA and incubated at 37°C for 20 min in the presence of $CaCl_2$. Enlargement of Figs. 4a, 5a, and 6a is × 20,000. Enlargement of Figs. 4b, 5b, and 6b is × 60,000.

FIG. 5. Thin-sectioned *E. coli* cells treated with Tris-EDTA and incubated at 37°C for 20 min in the presence of phospholipase A_2 and $CaCl_2$.

FIG. 6. Thin-sectioned *E. coli* cells treated with Tris-EDTA and incubated at 37°C for 20 min in the presence of phospholipase C and $CaCl_2$.

hydrolysis of membrane phospholipids by exogenous phospholipase A_2 and C and endogenous phospholipase A_1 and/or a lysophospholipase. The extent of degradation suggests that both phospholipase A_2 and C can penetrate the murein layer of *E. coli* and hydrolyze phospholipids in the cytoplasmic membrane. Electron micrographs show that even when there is extensive degradation of membrane phospholipids some sort of a barrier remains between the cytoplasm and the periplasmic space.

Earlier experiments on *Bacillus subtilis* (15) and *Bacillus megaterium* (J. A. F. Op den Kamp, unpublished observations) showed that in these organisms the murein layer surrounding the cell excluded phospholipases. The explanation for this difference might be that in *E. coli*, the murein layer is not thick enough to act as an effective barrier.

Our results on phospholipase C action are in agreement with the data obtained by Mavis *et al.* (21) on membrane preparations and lipid extracts of *E. coli* and indicate that the membrane proteins do not affect the accessibility of membrane phospholipids to this enzyme. The fact that cardiolipin, which is a good substrate for phospholipase A_2, is hardly degraded when this enzyme is added to intact cells indicates that cardiolipin may be located in the membrane in such a way that it is inaccessible to this enzyme. By contrast, phosphatidylethanolamine and phosphatidylglycerol are readily accessible to phospholipase A_2. The observation that phosphatidylglycerol is hardly degraded by phospholipase C is probably due to the fact that this enzyme preferentially hydrolyzes zwitter ionic phospholipids (17).

Patriarca, Beckerdite, and Elsbach (18) showed that endogenous lipolytic activities become unmasked after EDTA-lysozyme treatment of *E. coli* but not after EDTA treatment alone. Our results are in contrast with these observations. On the other hand, the nature of the unmasked enzymatic activities is similar. The pH profiles, substrate specificity, and inhibition by detergents of lipolytic activities which are found after our EDTA treatment are identical to those described by Patriarca *et al.* (J. A. F. Op den Kamp; unpublished results). Preliminary results suggest that a difference in time of harvesting might cause the observed discrepancy. Unmasking of these enzymes by EDTA alone may be found only in cells which are harvested in the logarithmic phase of growth.

Despite the almost complete conversion of phospholipids into lysophospholipids and fatty acids by phospholipase A_2 and diglyceride by phospholipase C, the cell does not disintegrate and large-molecular-weight material is retained within the cytoplasm. These results strongly suggest that the membrane has retained at least part of its structural integrity. The death of the cells, however, suggest that some functional integrity has been lost.

We suspect that the earlier observation (12) that phospholipase C causes lysis was due to the presence of another lytic enzyme in the phospholipase C preparation. Sphere formation (13) is caused not only by successive Tris-EDTA-phospholipase C treatment but also during the incubation of Tris-EDTA-treated cells at 37°C in the presence of $CaCl_2$. The biochemical background for this phenomenon remains obscure.

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