Effect of Ethylenediaminetetraacetate on Phospholipids and Outer Membrane Function in *Escherichia coli*

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Treatment of *Escherichia coli* K-12 strain S15, containing a normal amount of phospholipase A, with ethylenediaminetetraacetate (EDTA) resulted in an increase in sensitivity of the organism to actinomycin D. Strain S17, a mutant deficient in both detergent-resistant phospholipase A and detergent-sensitive phospholipase A, was considerably less sensitive to the antibiotic after the treatment. Both strains released lipopolysaccharide after EDTA treatment, indicating that this outer membrane component alone is not the barrier to actinomycin in these organisms. The phospholipase A-deficient strain released less alkaline phosphatase, a periplasmic enzyme. EDTA treatment of S15 resulted in the accumulation of free fatty acids, indicative of phospholipase A activation. Cells briefly treated with EDTA regained the barrier to actinomycin when incubated in growth media, and the cessation of the accumulation of free fatty acids was in approximate temporal agreement with restoration of the barrier. Cells in which phospholipase A was activated by brief exposure to EDTA synthesized relatively more phosphatidylethanolamine than did untreated cells in the initial period after dilution into growth media. These experiments suggest that the EDTA-induced loss of outer membrane barrier function of *E. coli* K-12 is mediated through the activation of phospholipase A.

One approach to understanding relationships between structure and functions in biological membranes involves the use of membrane perturbants, such as ethylenediaminetetraacetate (EDTA). Treatment of *Escherichia coli* with EDTA results in the release of lipopolysaccharide (LPS) and other outer membrane components (16, 17). Treatment of gram-negative bacteria with EDTA also results in an increased sensitivity to actinomycin D (16, 17), implying that the outer membrane normally excludes the antibiotic. It has been shown that treatment of *E. coli* with EDTA results in the activation of a phospholipase A (10), but the identity of the enzyme was not established. Phospholipase A enzymes are capable of deacylating phospholipids, and their activation frequently results in the accumulation of free fatty acids. Phospholipase A_1 is a phosphoglyceride 1-acylhydrolase, and phospholipase A_2 (formerly B) is a phosphoglyceride 2-acylhydrolase. An additional membrane perturbant that alters membrane function is the attachment of T4 phage and T4 ghosts to *E. coli* (9). T4 ghosts are devoid of DNA, and, because the particle itself does not enter the host, the functional changes observed must be a consequence of membrane alteration. We have shown that a phospholipase A is associated with T4 phage and ghosts (24) and that attachment of the ghost particles activates a host phospholipase A (5). Additionally, it has been demonstrated that the attachment of T2 phage (23) and T4 phage (Mizutani and Buller, unpublished data) to *E. coli* results in an increase in sensitivity of the host to actinomycin, much as was observed after EDTA treatment (16, 17). Furthermore, it has been suggested that a detergent-resistant phospholipase A which is compartmentalized in the outer membrane of *E. coli* is activated by phage infection and has a role in the dissolution of the outer membrane (12).

The mechanism whereby EDTA initiates the release of LPS and the accompanying change in permeability of the outer membrane has not been elucidated. One obvious possibility is that the chelation of divalent cations bridging adjacent LPS molecules, or LPS molecules to adjacent phospholipids, would result in repulsion between the negative charges, thus contributing to the expulsion of the LPS. Hydrophobic interactions between the phospholipids and the lipid A moiety of the LPS undoubtedly also contribute to the structure and stability of the outer membrane. It follows that deacylation of the phospholipids in the outer membrane could weaken these hydrophobic interactions, perhaps sufficiently to permit the release of LPS and/or lead to increased sensitivity to actinomycin.

MATERIALS AND METHODS

Bacteria. *E. coli* K-12(λ) strain S17 (*leu thr thi lac mal pldA* Pld2⁻), previously described by Nojimal et al. (8, 28), is deficient in both a detergent-resistant phospholipase A and a detergent-sensitive phospholipase A. Abe et al. (1) have determined the map position of the *pldA* gene for the detergent-resistant enzyme (phenotype designation, Pld1), compartmentalized in the outer membrane (3, 29), and have shown it to be distinct from an unmapped detergent-sensitive phospholipase (phenotype designation, Pld2) associated with the cytoplasmic membrane (8). The parent strain S15 (*leu thr thi lac mal* Pld1⁺ Pld2⁺) is considered to be wild type in regard to phospholipase A activities. Both S15 and S17 were obtained from S. Nojima. Alkaline phosphatase constitutive mutants of both strains arose spontaneously and were selected by the procedure of Torriani and Rothman (34).

Special chemicals. [1,2-¹⁴C]Sodium acetate (56.2 mCi/mmol) and [2-¹⁴C]uracil (50 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass. All reagents used in phospholipid and LPS extractions were reagent grade. Silica gel, Camag type DO without binder, was obtained from Arthur H. Thomas, Philadelphia, Pa. Aquasol, obtained from New England Nuclear Corp., and 3a70B complete counting cocktail, obtained from Research Products International Corp., Elk Grove Village, Ill., were used as scintillation fluids. Sigma 104 alkaline phosphatase substrate (*p*-nitrophenyl phosphate), EDTA, trizma base, and actinomycin D were purchased from Sigma Chemical Co., St. Louis, Mo.

Culture conditions. Tryptone broth containing 1% tryptone (Difco Laboratories, Detroit, Mich.) and 0.1 M NaCl was used for growth of bacteria. Overnight cultures were diluted 1:100 into fresh media, and the cultures were incubated at 37°C with aeration until cell density was about 2×10^8 cells per ml. In experiments in which the metabolism of phospholipids and LPS was examined, the growth media contained 0.4 μCi of sodium [¹⁴C]acetate per ml to label the acyl groups of these compounds.

Treatment with EDTA. A modification of the procedure of Leive (17) was used for treating cells with EDTA. The bacteria were collected by centrifugation at $5,000 \times g$ and 25°C for 10 min. After being washed two times with 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, pH 7.4, the cells were suspended in 0.12 M Tris-hydrochloride, pH 8.0, at a volume equivalent to one-tenth of the original culture volume. EDTA (final concentration, 0.5 mM) was added immediately, and the mixture was incubated with shaking at 37°C. After 2 min the mixture was diluted 10-fold with growth media or 0.12 M Tris-hydrochloride buffer, pH 8.0, both at 37°C, to terminate EDTA activity. Control cells were prepared in the same manner except that EDTA was omitted. To avoid the possibility of any reassociation of outer membrane components, we did not use Mg²⁺ to terminate EDTA activity. That EDTA activity is terminated by a 10-fold dilution into growth media was indicated by a gradual restoration of the barrier to actinomycin.

Phospholipid extraction and characterization. Bacteria containing phospholipids with acyl groups labeled by growth in media containing [¹⁴C]acetate were collected by centrifugation. Phospholipids and free fatty acids in both supernatant and precipitate fractions derived from 5 ml of culture were extracted by the procedure of Cronan and Wulff (6). The chloroform phases were concentrated to dryness in a stream of N₂, and the lipids were dissolved in 50 μl of CHCl₃ and used for thin-layer chromatography (TLC).

TLC was performed by a modification of the procedure of Skipski and Barclay (32). Plates were coated with Camag gel type DO without binder (0.5 mm thick), and 1.0 mM borate instead of water was used to prepare the slurry to facilitate the separation of phosphatidylethanolamine and phosphatidylglycerol (14). Plates were first developed to 16 cm in acetone–petroleum ether (1:3, vol/vol) and then placed in a vacuum desiccator for 15 min. Before development in chloroform–methanol–1.0 mM borate (65:25:4, vol/vol/vol), the TLC plates were exposed to 62% (wt/wt) H₂SO₄ for 45 to 60 min in a Camag Vario-KS chamber to establish a relative humidity of 18%. After development of the TLC plates, the lipids were detected by radioautography. The radioactivity in each spot was determined by scintillation spectrometry as previously described (5) except that 3a70B scintillation fluid was used. The identity of the individual phospholipids was established as previously described (30).

Assay for LPS release. The extent of LPS release from EDTA-treated cells was determined by the relative amounts of β-[¹⁴C]hydroxymyristic acid in hydrolysates of precipitate and supernatant fractions. β-Hydroxymyristic acid is unique to the lipid A moiety of LPS and therefore can be used as a marker for its release (15, 33). After EDTA treatment the cells were diluted into growth media and incubated for 10 min, followed by centrifugation at room temperature. Phospholipids and free fatty acids were then extracted from both supernatants and precipitates as described above. To determine the amount of LPS released into the supernatant, the water–methanol phase, which was obtained after the lipid extraction, was concentrated to dryness in a rotary evaporator. The dry residue was then taken up in methanol containing freshly generated HCl at a 2 N concentration, followed by incubation for 15 h at 80°C in sealed tubes. The precipitate fractions which were obtained after phospholipid extraction were hydrolyzed in the same way. After hydrolysis the fatty acids were extracted and fractionated by TLC, and the relative amounts of β-hydroxymyristic acid in each fraction were determined as previously described (15).

Effect of EDTA on sensitivity to actinomycin. The effects of EDTA treatment on the sensitivities of strains S15 and S17 to actinomycin D inhibition of RNA synthesis were compared by the method described by Leive (17). After being washed and suspended, the cells were treated with EDTA as described above. After EDTA treatment the cells were diluted 10-fold into growth media and incubated for 3 min at 37°C, and then actinomycin D was added to a final concentration of 10 μg/ml. At 1 min after addition of the antibiotic, 0.5 μCi of [¹⁴C]uracil was added per ml of mixture. The samples were incubated for an additional 2 min with shaking at 37°C, and 0.2-ml portions were removed to tubes containing 2.5 ml of cold 5%

trichloroacetic acid. The acid precipitates were collected on membrane filters (Millipore Corp., Bedford, Mass.) and washed with 8 ml of distilled water. The incorporation of radioactivity into the precipitated cells was determined by scintillation spectrometry of the filters by using Aquasol as the scintillation fluid.

Alkaline phosphatase release. The rates of release of alkaline phosphatase, a periplasmic enzyme (21), from strains S15 and S17 were compared as follows. Spontaneous mutants constitutive for alkaline phosphatase synthesis were treated with EDTA as described above. After EDTA treatment they were diluted 10-fold into 0.05 M Tris-hydrochloride, pH 8.0, and incubated for 10 min at 37°C. The cells were then collected by centrifugation at room temperature. The supernatant was held for the determination of the amount of alkaline phosphatase activity. To quantitate the amount of alkaline phosphatase retained in the cells, the precipitates were resuspended at a density equivalent to the density before centrifugation, and toluene (final concentration, 1%) was added. The amount of alkaline phosphatase activity in the super-. natant and in the resuspended cells was determined by a modification of the procedure of Malamy and Horecker (22). Assay mixtures contained 0.75 ml of toluene-treated cells or supernatant fraction and 2.25 ml of 1.0 M Tris-hydrochloride, pH 8.0, containing 1.0 mM p-nitrophenyl phosphate. Rate of hydrolysis was assayed with a Gilford recording spectrophotometer. A unit of enzyme activity was defined as the quantity of enzyme activity required to produce a change of 0.1 absorbancy unit at 420 nm per min at 29°C.

RESULTS

Phospholipase A and sensitivity to actinomycin D. The effect of EDTA on the sensitivity of strains S15 and S17 to actinomycin D is shown in Fig. 1. If sensitivity to the antibiotic after EDTA treatment is mediated through activation of phospholipase A, the cells deficient in the outer membrane phospholipase A should remain relatively insensitive. Fig. 1A indicates that incorporation of [^{14}C]uracil into trichloroacetic acid-precipitable material was severely inhibited in S15. Similar treatment of the phospholipase A-deficient strain S17 did not result in significant inhibition (Fig. 1B).

The effect of actinomycin on the viability of untreated cells was examined because mutants with increased sensitivity to the antibiotic have been isolated (13, 31) and the validity of the experiments described above is dependent upon one strain not being inherently more sensitive than the other to the antibiotic at the concentrations used. Exposure of both strains to 10 µg of actinomycin per ml had no effect on survival of either; at 100 µg/ml S15 was somewhat more sensitive. The growth of either strain was not inhibited after suspension in 0.12 M Tris buffer (pH 8.0) and exposure for 2 min to 0.5 mM EDTA. When treatment with 0.5 mM EDTA
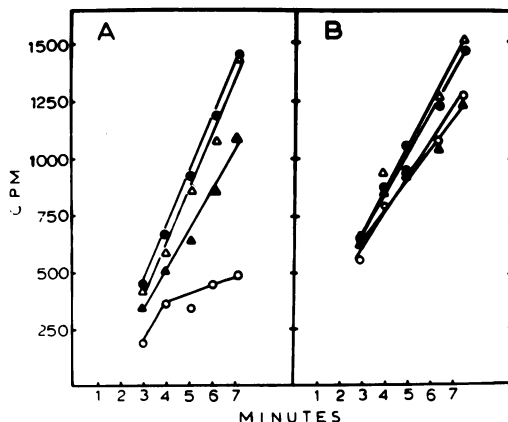

FIG. 1. *Sensitivity to actinomycin D. Uptake of [^{14}C]uracil into acid-insoluble material was compared in treated and untreated strains S15 (A) and S17 (B). Cultures were prepared and treated with EDTA as described in the text. At 3 min after EDTA treatment cells were diluted into growth media at 37°C containing 10 µg of actinomycin D per ml (zero time). After 1 min [^{14}C]uracil (0.5 µCi/ml) was added, and samples were assayed as indicated in the text. Symbols: △, control (not treated with EDTA or actinomycin); ▲, EDTA treatment only; ●, actinomycin treatment only; ○, EDTA treated and exposed to actinomycin D.*

was prolonged to 5 min or if 1.0 mM EDTA was used, there was a pronounced decrease in viability of both strains. Accordingly, the inhibitory effect of actinomycin D was measured only under the nonlethal conditions of EDTA treatment.

Phospholipase A and release of alkaline phosphatase. Alterations in the nonspecific permeability barrier function of the outer membrane might be expected to have an effect on the retention of periplasmic enzymes. These enzymes normally can be released by an osmotic shock procedure which also involves EDTA treatment (2, 25). *E. coli* cells with defective LPS have been found to excrete penicillinase, an enzyme found in the periplasmic space of penicillin-resistant mutants (11). To determine whether phospholipase A could be implicated in the EDTA-catalyzed release of periplasmic proteins, we compared the release of alkaline phosphatase from S15 and S17 mutants which were constitutive for the synthesis of this enzyme. The results of such experiments (Table 1) indicate that after EDTA treatment a greater percentage of total cellular alkaline phosphatase was released from S15. The extent of release was less than that usually observed after osmotic shock (25), presumably because the conditions were milder.

LPS release. If treatment of strain S17 with EDTA resulted in the release of LPS, then it could be concluded that LPS alone does not constitute the barrier to molecules such as actinomycin. LPS release was examined by quantitating the amount of β-hydroxymyristic acid in hydrolysates of supernatants of EDTA-treated S15 and S17. β-Hydroxymyristic acid is found uniquely in the lipid A moiety of LPS. Table 2 indicates that EDTA treatment resulted in the release of nearly the same amount of LPS from both S15 and S17.

Effect of EDTA on phospholipid metabolism. Strains S15 and S17 were grown in media containing [^{14}C]acetate to label the acyl groups of phospholipids. After washing and suspension in 0.05 M Tris-hydrochloride, pH 8.0, the cells were treated for 2 min with 0.5 mM EDTA. Calcium chloride was then added to a final concentration of 5 mM, and after a 10-min incubation phospholipids were extracted and characterized. A decrease in the relative amount of phosphatidylethanolamine and the accumulation of free fatty acids were observed only in the phospholipase A-containing strain S15 (Table 3). The accumulation of labeled free fatty acids is indicative of phospholipase A activation and thus confirms a report (10) that such an enzyme

TABLE 1. *Effect of EDTA on alkaline phosphatase release[a]*

Treatment	% Alkaline phosphatase released into the supernatant by strain:[b]	
	S15	S17
Control[c]	3.2	4.6
EDTA treated	26.3	12.5

[a] EDTA treatment of alkaline phosphatase constitutive mutants of the phospholipase-deficient strain S17 and its wild-type parent, S15, and assay of alkaline phosphatase activity are described in the text.

[b] Alkaline phosphatase in supernatant is expressed as the percentage of total cellular enzyme activity.

[c] EDTA omitted.

TABLE 2. *Effect of EDTA on LPS release[a]*

Treatment	β-Hydroxymyristate in supernatant with strain	
	S15	S17
Control[b]	3.1	5.0
EDTA treated	19.7	17.8

[a] Cells were treated with EDTA, and the amount of LPS released was quantitated as the amount of radioactivity in β-hydroxymyristic acid relative to the total activity (in supernatant and precipitate). Procedures are outlined in the text.

[b] EDTA omitted.

TABLE 3. *Effect of EDTA treatment on phospholipid composition of E. coli[a]*

Treatment	% Of total free lipid			
	Free fatty acids[b]	Cardiolipin	Phosphatidylethanolamine	Phosphatidylglycerol
S15				
Untreated[c]	0.6	12.9	73.1	12.8
EDTA treated	8.2	14.4	67.6	9.5
S17				
Untreated[c]	0.5	13.6	72.6	13.3
EDTA treated	0.6	16.2	73.6	8.4

[a] Acyl groups were labeled by growth in media containing [^{14}C]acetate. At 10 min after the addition of 5 mM $CaCl_2$ to stop EDTA treatment, phospholipids were extracted and fractionated as described in the text.

[b] Not esterified; migrated with or near the solvent front in TLC.

[c] Control cells, not treated with EDTA but suspended in 0.12 M Tris-hydrochloride, pH 8.0.

is activated by EDTA. In these experiments the relative amount of cardiolipin was greater than has been previously reported (5) and appeared to occur as a consequence of the conversion of phosphatidylglycerol to cardiolipin. This process is independent of phospholipase A, and we consistently observed it when cells were suspended in Tris buffer.

Phospholipid metabolism after EDTA treatment. Leive (17) has reported that cells regain their barrier to actinomycin when they are incubated in growth media after EDTA treatment. Similar results were obtained with strain S15 (Fig. 2). Within a one-generation time period the cells no longer were sensitive to the antibiotic. If the EDTA-catalyzed decrease in barrier function is related to phospholipid deacylation in the outer membrane, then it could be expected that this process would be brought under control at a time which correlates with the reacquisition of resistance. This was examined by measurement of the accumulation of free fatty acids derived from phospholipids labeled before EDTA treatment. Figure 3 shows that the accumulation of free fatty acids was most rapid during the initial 40 min after EDTA treatment, after which the process subsided.

An examination of the relative amounts of the individual phospholipid classes synthesized during the recovery period after EDTA treatment indicated that more phosphatidylethanolamine was synthesized by treated than by control cells (data not shown). This is expected because the
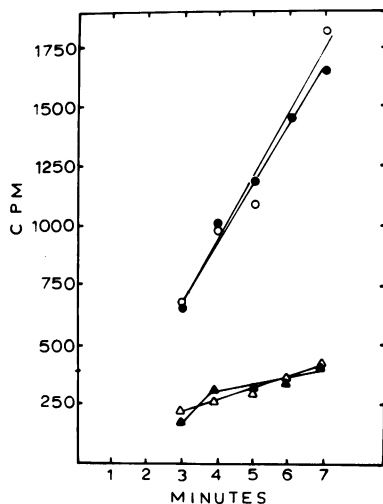
FIG. 2. *Regain of resistance of EDTA-treated, phospholipase A-positive cells to actinomycin. Strain S15 was prepared and treated with EDTA as described in the text. After treatment cells were diluted into growth media and incubated with shaking at 37°C for 3 (△), 15 (▲), or 35 (●) min before the addition of 10 µg of actinomycin D per ml (zero time). [^{14}C]uracil (0.5 µCi/ml) was added to each tube at 1 min after the antibiotic was added, and its incorporation was measured as described in the legend to Fig. 1. ○, control (no actinomycin present).*

primary target of the outer membrane phospholipase A of S15 is phosphatidylethanolamine. Such experiments, however, remain inconclusive because the control cells demonstrated an unusual pattern of synthesis. Normally phosphatidylethanolamine comprises about 70% of the total phospholipids in exponentially growing cells, as measured after a 2-h growth period in media containing [^{14}C]acetate (Table 3). However, these experiments, in which phospholipid synthesis was measured during the initial 10 min after suspension in media with [^{14}C]acetate, consistently showed in control cells that phosphatidylethanolamine represented only 33% of phospholipids, as opposed to 63% in the EDTA-treated cells. Because the cells were physically manipulated before EDTA treatment, the altered phospholipid synthesis pattern in control cells may be a consequence of perturbation incurred during centrifugation.

DISCUSSION

The establishment of biochemical function(s) of an enzyme is often facilitated by the availability of a mutant deficient in that enzyme. We have utilized a mutant which is deficient in both the detergent-resistant and detergent-sensitive phospholipase A activities to examine the effects

of conditions which normally activate the enzyme on the function of the outer membrane of *E. coli* as a nonspecific permeability barrier. These experiments suggest that the increase in permeability of the outer membrane after treatment with EDTA may be explained in part by the activation of phospholipase A. Thus *E. coli* strains which·contain the detergent-resistant phospholipase A in the outer membrane become more sensitive to actinomycin than do mutants deficient in this enzyme. The EDTA-stimulated release of alkaline phosphatase, a periplasmic enzyme, is more extensive in cells which contain the outer membrane phospholipase A.

The activation of phospholipase A, however, cannot alone account for the EDTA-induced release of LPS, because release of this macromolecule was observed in both the wild type and the phospholipase A-deficient mutant. It has been reported that the material released by EDTA could be separated into F1 and F2 fractions (18). F1 consisted of LPS, protein, and lipid, whereas F2 was LPS. In their mutants which did not become sensitive to actinomycin after EDTA treatment, less F2 was released, whereas equivalent amounts of F1 were released (35). More recently, it was shown that resistance could be partially restored and that the efficiency of reconstitution was greatest if both F1 and F2 were used (4). Because both S15 and S17 released similar amounts of LPS, we did not attempt to fractionate it into F1 and F2 components.

The mechanism whereby EDTA activates phospholipase A is not known. It has been reported that the outer membrane phospholipase A requires Ca^{2+}, and in vitro assays in which EDTA is continually present show that the enzyme is inhibited (27). Because the detergent-resistant phospholipase A can be expected to be an allotopic enzyme, i.e., one in which activity depends on a hydrophobic environment, it is likely that a perturbation in the hydrophobic portion of the membrane might activate it. A tentative explanation thus could be that EDTA chelates divalent cations from the outer membrane, resulting in the expulsion of negatively charged LPS. This would alter the hydrophobic environment about phospholipase A, leading possibly to its activation and concomitant deacylation of neighboring phospholipids.

A potential flaw in the above rationale is that S15 and S17 may differ in other properties exclusive of phospholipase A. They were isolated by nitrosoguanidine mutagenesis (8, 28), and it is therefore likely that they contain unscored mutations. However, these strains do not differ in the efficiency of adsorbing T4 phage, indicating that LPS is normal at least as a phage

receptor, and no differences in outer membrane proteins have been found (Hardaway and Buller, unpublished data). Additionally, they are not more sensitive than other *E. coli* K-12 strains to lysis by anionic detergents, e.g., sodium dodecyl sulfate (Buller, unpublished data). Doi and Nojima (7) extensively compared properties of both and did not find differences in growth and lipid composition, nor have we observed any reversion of S17 to its parental strain. Perhaps the most convincing evidence that unscored mutations are not involved here lies in the observation that neither EDTA nor actinomycin D (10 µg/ml) had an effect on the viability of S15 and S17. At higher concentrations of actinomycin both were sensitive, indicating that an unscored mutation(s) does not make the phospholipase-deficient cells permanently resistant. Also, EDTA releases LPS from both strains (Table 2), indicating that an unscored mutation does not preclude outer membrane alteration. Thus the effects of EDTA and actinomycin treatment do not seem to be altered by unscored mutations, and sensitivity to the antibiotic is apparent only when phospholipase A is present.

Cells may become sensitive to actinomycin within 15 s after the addition of EDTA. From 35 to 50% of the total eventual LPS release may occur during that time, and, when used briefly, EDTA apparently affects only the outer membrane (17). The measurable accumulation of free fatty acid, however, does not occur this rapidly (Fig. 3). This, however, does not exclude the involvement of phospholipase A because the deacylation may occur only in those domains in which the enzyme is embedded and thus repre-

sent only a relatively small part of the outer membrane. The phospholipid content of the cytoplasmic membrane and that of the outer membrane are approximately the same (20). Additionally, the amount of free fatty acid measured is expressed as the percentage of total phospholipid, whereas the change in question is localized in the outer membrane. Finally, the observed free fatty acid accumulation represents a minimum level because, once released, the free fatty acids may be transported back into the cell and thus become subject to β-oxidation, or, as has been shown recently, acylation may occur in the outer membrane (36).

Nikaido (26) has suggested that molecules with a relatively high partition coefficient in 1-octanol–0.05 M sodium phosphate, pH 7, may enter bacterial cells by passive diffusion through hydrophobic areas of the outer membrane. This was supported by the increased sensitivity of deep rough mutants of *Salmonella typhimurium* to certain antibiotics, including actinomycin. The deep rough mutants have LPS with much shortened saccharide chains, and, because the number of lipid A subunits is not decreased, the overall hydrophobic character of the membrane is increased. It was also suggested that the outer membrane is further modified by an increase in the phospholipid content of the outer face of the membrane. EDTA treatment, however, results in the release of LPS, phospholipid, and protein (16, 17), as well as phospholipid deacylation. Similarly, the attachment of T4 phage to *E. coli* is known to cause a release of outer membrane components (19) and is accompanied by a temporary increase in sensitivity to actinomycin (23; Mizutani and Buller, unpublished data). Thus, both physical and chemical modifications of the permeation barrier occur as a consequence of EDTA treatment.
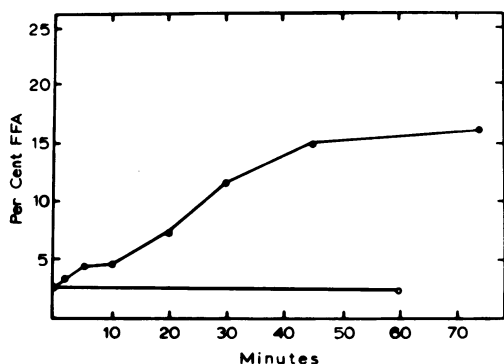
FIG. 3. *Free fatty acid (FFA) formation after EDTA treatment of S15. The acyl groups of phospholipids were labeled by growth of the wild-type strain in media containing [¹⁴C]acetate. At various times after the treatment of the cells with EDTA, samples were removed for phospholipid extraction and fractionation as outlined in the text. Symbols: ○, untreated; ●, EDTA treated.*

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