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Effects of Fatty Acid Substitution on the Release of Enzymes by Osmotic Shock

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The release of enzymes by osmotic shock from *Escherichia coli* strain 30E, an unsaturated fatty acid auxotroph, was examined in culture supplemented with either *cis*- or *trans*-unsaturated fatty acids. Cultures grown in oleate-supplemented medium release a large fraction of the total cyclic phosphodiesterase, acid hexose phosphatase, and 5'-nucleotidase following osmotic shock. Cultures grown in elaidate-supplemented medium release much less of these same enzymes after shock treatment. Cultures grown with either supplementation show total release of these enzymes upon conversion to spheroplasts, demonstrating that the enzymes are in the periplasmic space in both cases. Cultures grown with either oleate or elaidate as fatty acid source were washed and suspended in medium containing the other isomer. The change from oleate to elaidate resulted in a rapid decrease in ability of the cells to release the three enzymes after osmotic shock so that within a 25% increase in cell mass the culture responded to osmotic shock as would a culture grown overnight in elaidate-supplemented medium. The reverse experiment resulted in a gradual increase in the ability of the cells to respond to osmotic shock. The outer membrane of *E. coli* is altered by the incorporation of elaidate, as indicated by electron microscopic data.

Auxotrophic strains of *Escherichia coli* (7, 10, 13, 26, 27, 30) requiring unsaturated fatty acids have been isolated and studied in a number of different laboratories. These mutants have been utilized by Schairer and Overath (26) and Wilson et al. (30) to demonstrate that the effect of membrane lipid composition on sugar transport is related to the fatty acid supplied in the growth medium and is independent of the sugar transport system studied. Moreover, newly synthesized membrane proteins appear to associate preferentially with newly formed membrane. Although the M protein is incorporated into the membrane in these mutants, the induction of β-galactoside transport is prevented by withholding the required fatty acid during induction (13).

We report here the effects of growth on *cis*- and *trans*-unsaturated fatty acids in such a mutant on the selective release of proteins by osmotic shock and on the transport of amino acids. Growth in media containing *trans*-unsaturated fatty acids was found to cause changes in the release of several enzymes, as well as changes in the morphology of this mutant. The data are discussed in terms of the effects of lipid composition on the structure and function of the cell surface.

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MATERIALS AND METHODS

Bacterial strains and media. Strain 30E, an unsaturated fatty acid auxotroph of *E. coli* K-12, was generously provided by C. F. Fox (31). Strain 30E can fulfill its unsaturated fatty acid requirement with elaidate, unlike its parent, strain 30$^-$, which cannot utilize *trans*-unsaturated fatty acids. Cultures were grown in a minimal medium consisting of medium A (3) and 1% casein hydrolysate (Nutritional Biochemicals Corp.) with shaking at 37 C. Fatty acids were added at 0.02% and were solubilized by the addition of 0.5% Triton X-100 (Rohm & Haas Co.). Cells used in this study were harvested in the exponential phase of growth (5×10^8 to 1×10^9 cells/ml).

Chemicals. ^{14}C-arginine and ^{14}C-glycine were obtained from New England Nuclear Corp., Boston, Mass., and had specific radioactivities in excess of 200 mCi/mmole. Oleic (*cis*-Δ^9-octadecenoic) acid, elaidic (*trans*-Δ^9-octadecenoic) acid, *cis*-vaccenic (*cis*-Δ^{11}-octadecenoid) acid, and *trans*-vaccenic (trans-Δ^{11}-octadecenoic) acid were obtained from either Sigma Chemical Co., St. Louis, Mo. or the

Hormel Institute, Austin, Minn. Authentic 2-keto-3-deoxyoctulosonic acid was obtained from Edward Heath. All other chemicals were analytical grade and were purchased from commercial sources.

Assay for transport activity. Cells were harvested during the exponential phase of growth and washed twice at 23 C in minimal medium lacking fatty acids. Transport assays were performed as described previously (25) with the following exceptions. Aminooxyacetic acid, previously used to prevent the decarboxylation of arginine, was not used in this study because it has no effect on the initial rate of arginine transport. The temperature of the transport assays was varied by adjusting the cells, media, and pipettes to the desired temperature in a constant temperature water bath (Neslab Instruments, Portsmouth, N.H.).

Osmotic shock. Osmotic shock was performed according to the procedure of Neu and Heppel (22).

Preparation of spheroplasts. Cells were harvested during the exponential phase of growth, washed twice with either 0.154 M NaCl or 0.01 M tris(hydroxymethyl)aminomethane (Tris) containing 0.03 M NaCl, *p*H 8, at 23 C. The cells were suspended in 80 volumes (w/v) of 0.033 M Tris, *p*H 8, containing 20% sucrose. Ethylenediaminetetraacetic acid (EDTA), *p*H 7, was added to give a final concentration of 1 mM, immediately followed by the addition of lysozyme to the desired concentration which varied from 1 to 10 μg/ml. The mixture was swirled on a rotary shaker at 23 C. Portions (0.1 ml) were diluted 10-fold with water at various times, and the optical density was measured at 600 nm, indicating the extent of lysis, which depends on the degree of spheroplast formation. For the study of enzyme release, the spheroplasts were harvested 10 min after the addition of lysozyme by centrifugation in the cold at 7,000 × *g* for 10 min. The supernatant fraction was carefully decanted, and the pelleted spheroplasts were lysed by suspension in 80 volumes (w/v) of cold water, followed by vigorous agitation.

Enzyme assays. β-Galactosidase was assayed by measuring the rate of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis (19). One unit of activity represents a change of absorbancy of 1.0 measured at 410 nm at 23 C.

For assay of cyclic phosphodiesterase, the reaction mixture (0.1 ml) contained 0.2 μmole of uridine-2',3'-cyclic phosphate, 0.5 μmole of $MgCl_2$, 0.1 μmole of $CoCl_2$, 5.0 μmoles of sodium acetate buffer, *p*H 5.8, and excess purified alkaline phosphatase from *E. coli* (15). For assay of acid hexose phosphatase, the reaction mixture contained, in 0.1 ml, 0.4 μmole of glucose-6-phosphate, 10 μg of bovine serum albumin, and 10 μmoles of sodium acetate buffer, *p*H 5.8 (21). For determination of 5'-nucleotidase activity, the reaction mixture (0.1 ml) contained 0.5 μmole of 5'-adenosine monophosphate, 2 μmoles of $CaCl_2$, 0.6 μmole of $CoCl_2$, 10 μg of bovine serum albumin, and 10 μmoles of sodium acetate buffer, *p*H 5.8 (21). All three enzyme assays were performed by incubating the reaction mixture for 20 min at 37 C after the addition of enzyme followed by the determination of inorganic phosphate. P_i was measured according to the method of Chen et al. (6) as modified by Ames and Dubin (1).

The 5'-nucleotidase inhibitor was measured by diluting the enzyme 16-fold with water, incubating at 37 C for 90 min, and assaying for 5'-nucleotidase activity (9). This treatment inactivates the inhibitor, so that the increase in enzyme activity gives a measure of the amount of inhibitor present.

For the determination of adenosine triphosphatase activity, the reaction mixture (0.3 ml) contained 1.5 μmoles of adenosine triphosphate (ATP), *p*H 7, 0.6 μmole of $MgCl_2$, and 30 μmoles of Tris buffer, *p*H 9 (11). The reaction was carried out at 37 C for 20 min after the addition of enzyme. P_i was determined by the method of Fiske and Subba Row (12).

In many cases, the activity expressed by whole cells was used as the control value. In most cases, this value was between 70 and 90% of the extract activity. But, in some cases, the activity of whole cells was much greater than the extract value. This variation may be a function of the effectiveness of the preparation of extract. However, denaturation may also be a factor. In this respect, the activity of whole cells is a more reliable measure of the content of periplasmic enzymes that is total activity. The methods used for preparation of the extracts included treatment with chloroform and sodium dodecyl sulfate (20); treatment with lysozyme, EDTA, and chloroform; sonic treatment; sonic treatment after lysozyme and EDTA treatment; and treatment with toluene. Toluene treatment was later found to give the most consistent results.

Electron microscopy. Well-washed cells were fixed with 5% glutaraldehyde and postfixed with 1% osmium tetroxide, followed by dehydration with solution of increasing ethanol concentration according to the method of Telford and Matsumura (28). The cells were embedded in Epon-Araldite resin. Sections were cut on a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with an AEL EM6B electron microscope.

Other methods. 2-Keto-3-deoxyoctulosonic acid was determined according to the method of Weissbach and Hurwitz (29) as modified by Osborn (23). Samples of shock fluids (50 and 100 μg) were electrophoresed on polyacrylamide disc gels according to the method of Davis (8). Sensitivity to actinomycin D was examined by the method of Leive (17). Sensitivity to sodium dodecyl sulfate was determined by incubating the cells at 37 C for 20 min in the presence of 0.1 μg, 0.5 μg, and 1.0 μg of detergent in a total volume of 1.0 ml. The decrease in absorbancy at 600 nm is a measure of the sensitivity. Sensitivity to sodium deoxycholate was determined by measuring the generation time of the cells in media containing 0.2%, 0.5%, and 1.0% sodium deoxycholate. Turbidity was measured with a Klett-Summerson colorimeter (no. 66 red filter). Protein concentrations were determined by a micromodification of the method of Lowry et al. (18) with bovine serum albumin as a standard.

RESULTS

Effects of fatty acid supplement on the release of enzymes by osmotic shock. The effects of osmotic shock on cells from cultures

of strain 30E grown in media supplemented with either oleate (oleate cells) or elaidate (elaidate cells) are given in Table 1. Untreated controls of the two cultures contain comparable amounts of cyclic phosphodiesterase and acid hexose phosphatase. The oleate cells respond to osmotic shock by releasing the bulk of their activities into the shock fluid. This response is similar to that of prototrophic strains of *E. coli* (2). Elaidate cells have a greatly reduced response to osmotic shock, releasing only 20 to 30% as much of the same enzymes as the oleate cells.

The loss of ability to respond to osmotic shock may be due to the *trans*-double bond of elaidate, because cells grown in the presence of *trans*-vaccenate show a similar effect (Table 2). By contrast, the *cis*-vaccenate-grown cells show normal release of cyclic phosphodiesterase, acid hexose phosphatase, and 5'-nucleotidase.

Cells grown with *trans*-unsaturated fatty acids showed no more release of β-galactosidase (Tables 1 and 2) than did cells grown with *cis*-unsaturated fatty acids; in all cases no more than 2% of the β-galactosidase was found in the shock fluid. This indicates that enzymes are still being released in a selective manner. In this regard, it is interesting to note that the osmotic-shock procedure caused the release of 4 to 5% of the total cellular protein in cultures supplemented with either *cis*- or *trans*-unsaturated fatty acids, 18:1. Thus, the large decrease in release of these periplasmic enzymes is not part of a general effect. The crude shock fluids of oleate and elaidate cells show many identical bands after electrophoresis on polyacrylamide disc gels with some differences in intensities among the bands and with a few clear-cut differences. No attempt has been made to determine the enzymatic activity (if any) of the bands which appear on gels from elaidate shock fluid but not on those from oleate shock fluid. Although this study was limited to the three enzymes listed in Table 2, it seems unlikely that the only differences between cells grown with *cis*- or *trans*-fatty acids would be in three randomly chosen enzymes.

TABLE 1. *Effect of fatty acid supplement on release of enzymes from strain 30E*[a]

Fatty acid supplement	Enzyme	Untreated control (units/mg)	Release (%)[b]
Oleate	Cyclic phosphodiesterase	1.68	79
	Acid hexose phosphatase	2.26	85
	β-Galactosidase	17.5	1
Elaidate	Cyclic phosphodiesterase	1.96	27
	Acid hexose phosphatase	2.45	16
	β-Galactosidase	21.6	1

[a] Cells were grown and shocked, and the enzyme assays were performed as described. Control extracts of unshocked cells were prepared by addition of two drops of chloroform and three drops of 0.25% sodium dodecyl sulfate/ml of cells (12.5 mg wet weight). The suspension was immediately and vigorously mixed by vortexing, followed by incubation at 37 C for 15 min (20).

[b] These values are the average of several experiments. Individual values varied as much as 10% in either direction from the indicated percentages.

TABLE 2. *Comparison of effects of cis- and trans-fatty acid supplements on release of enzymes by osmotic shock in strain 30E*[a]

Enzyme	Release (%)			
	Oleate	Elaidate	*cis*-Vaccenate	*trans*-Vaccenate
Expt 1				
Cyclic phosphodiesterase	114	38	94	25
Acid hexose phosphatase	69	21	40	11
Expt 2				
5'-Nucleotidase	28	0	30	0
β-Galactosidase	2	1	2	1

[a] The shock procedure and enzyme assays were performed as described. Experiment 1: the percentage release was calculated from the ratio of the enzyme activities of shock fluids and the enzyme activities expressed by whole, unshocked cells. Experiment 2: control values were determined by addition of 25 μg of lysozyme and 1 nmole of EDTA/ml of cells (12.5 mg wet weight) followed by incubation for 5 min at 37 C. Three drops of chloroform were added with vigorous mixing, and the suspension was incubated for an additional 10 min at 37 C.

Other effects of fatty acid supplementation. It has been shown that the Arrhenius plots for sugar transport in *E. coli* K-12 are biphasic (26, 30). The transition temperature (that temperature at which the slope changes) is independent of the transport system and depends solely on the fatty acid supplement. We have extended this observation to amino acid transport. Two transport systems were investigated: (i) the arginine specific system (25) which is not inhibited by other amino acids and which is reduced by more than 95% by osmotic shock, the reduction in transport being accompanied by release of an arginine specific binding protein; and (ii), the glycine transport system which is not affected by osmotic shock. Arrhenius plots for the two transport systems are shown for both oleate and elaidate-supplemented culture (Fig. 1). Here too, the transition between the slopes is independent of the system studied, occurring at 30 C for elaidate-supplemented cultures and at 13 C for oleate-supplemented cultures. This indicates that the mobility of the carrier through the membrane depends on the degree of ordered structure of the membrane, even in the

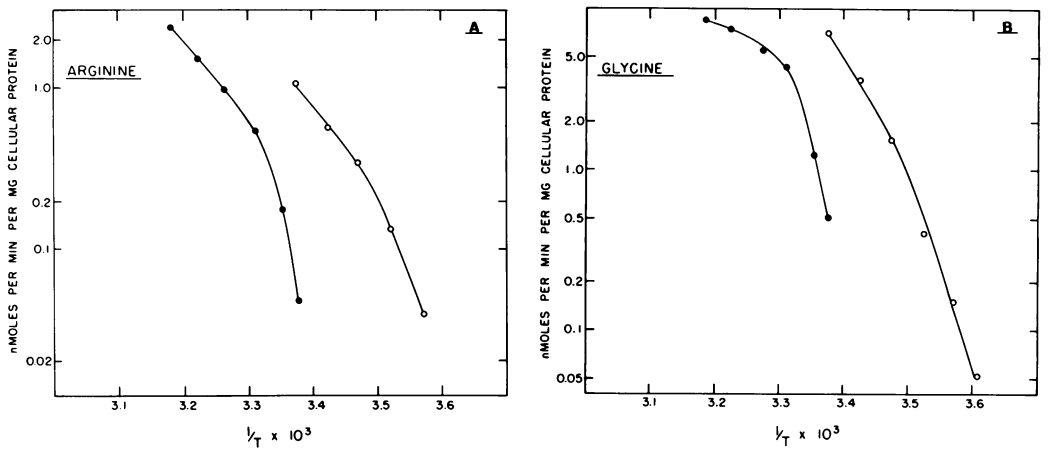

FIG. 1. *Temperature dependence of arginine and glycine transport. Oleate- (○) and elaidate- (●) grown cells were washed three times with minimal medium and then incubated for 15 min at 37 C in the presence of 0.2% glucose. A portion of these cells was then incubated for 5 min at the temperature used for assay in the presence of 80 μg of chloramphenicol per ml and 20 mM glucose. Assays for arginine (A) and glycine (B) transport were then performed at the desired temperature.*

case of arginine transport where the carrier is presumably shock releasable.

The first stage of the osmotic-shock procedure is carried out at 23 C and the second stage at 4 C. A transition temperature of 13 C for oleate-supplemented cells indicates that the lipid structure of the membrane is largely liquid at 23 C and primarily crystalline at 4 C. Cells supplemented with elaidate have a transition temperature of 30 C suggesting lipids in a highly ordered structure at both 23 and 4 C. To test whether the differing responses to osmotic shock were due to the degree of fluidity of the membrane during the various stages of shock, two variations in the shock procedure were made. In the first variation the initial washings and the first stage were at 37 C and the second stage at 4 C. Under these conditions, both sets of cultures would be above their transition temperature during the first step and below during the second. In the second variation, the whole shock procedure was at 4 C, and both sets of cultures were below the transition temperature for the whole time. In neither case was there release of any protein for either oleate or elaidate supplemented cultures.

Kinetics of release. Because the release of the three enzymes appears to depend on the nature of the unsaturated fatty acid in the membrane, it was of interest to determine how much oleate in the membrane needed to be replaced by elaidate before the membrane showed the characteristics of an elaidate membrane. As shown in Fig. 2A, within a quarter of a doubling of cell mass the response of the cells to osmotic shock fell nearly to a minimum. At the same time, the activity expressed by unshocked cells dropped only slightly. From one to three doublings, the activity expressed by the cells dropped to about 30% of the initial activity. Assays of toluenized cells confirmed that the total enzyme level decreased correspondingly. This decrease (although reproducible) is unexplained, because overnight cultures of elaidate cells (five to six doublings in elaidate medium) exhibited activities comparable to overnight cultures of oleate cells. Heated shock fluid showed only a slight increase in 5'-nucleotidase activity, indicating the results were not due to release of inhibitor.

In the reverse experiment (Fig. 2B) a culture of elaidate-supplemented cells was washed and suspended in oleate-containing medium. There was no increase in the release of 5'-nucleotidase after 0.25 doubling; there was a gradual increase until one full doubling, after which the release remained constant. The activity expressed by the control cells and the total activity of cells treated with toluene increased gradually, but the rate and magnitude of the increase in switching from elaidate to oleate was not as great as the rate and magnitude of the decrease in total activity in switching from oleate to elaidate. The results in both cases were similar for acid hexose phosphatase and cyclic phosphodiesterase (not shown).

Localization. The release of enzymes by osmotic shock has been cited as an indication of surface localization. It was necessary, there-

fore, to determine whether the effect of elaidate was due to the removal of the enzymes from the cell surface to the intracellular space or to a tighter association with the membrane. That the amount of enzyme activity shown by whole cells was not affected by elaidate supplementation made the latter hypothesis more likely. Another criterion for surface localization is the release of enzymes into the medium when cells are converted into spheroplasts. The results of such an experiment are given in Table 3. For each of the three enzymes tested nearly all of the activity was released into the medium upon conversion to spheroplasts.

The enzymes themselves are probably in their normal surface location in elaidate-grown cells because they can be assayed in whole cells and can be released during formation of spheroplasts. The abnormal response to osmotic shock must be due to some other change, possibly to a change in the outer membrane, the inner membrane, or both.

Effects of fatty acid supplementation on the outer membrane. Although the exact mechanism of the response to osmotic shock is not known, it is believed that many of the effects are due to changes in the outer membrane (2). Up to 50% of the outer membrane lipopolysaccharide is released during the EDTA treatment of stage 1. At the same time,

TABLE 3. *Spheroplast formation and release of enzymes from strain 30E*[a]

Enzyme	Fatty acid supplement	Units/mg of protein (control)	Percent of control	
			Spheroplast medium	Spheroplast lysate
5'-Nucleotidase	Oleate	7.68	92	8
	Elaidate	7.60	104	5
Acid hexose phosphatase	Oleate	2.22	70	7
	Elaidate	2.28	77	6
Cyclic phosphodiesterase	Oleate	2.68	180	12
	Elaidate	2.94	122	16

[a] Cells were converted to spheroplasts and enzyme assays were performed as described. A small sample of cells was withdrawn as a control before the addition of EDTA and lysozyme. Control activity refers to the activity expressed by whole cells.

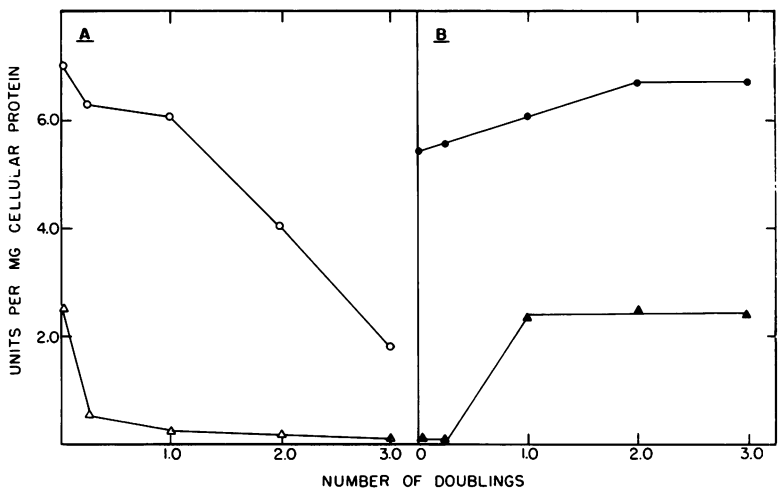

FIG. 2. *Effect of changeover in fatty acid supplement on release of enzymes from strain 30E. Cells were grown to mid-exponential phase in medium A containing 0.02% of either oleate or elaidate. Cells were then washed several times with 0.01 M Tris, pH 8, containing 0.03 M NaCl to remove all external fatty acid. Oleate-grown cells were resuspended in medium containing 0.02% elaidate, whereas elaidate-grown cells were resuspended in medium containing 0.02% oleate. The optical density (OD_{600}) of the resuspended cells was measured. A small sample of cells was withdrawn for the zero-time point. Growth was then allowed to continue. When the starting OD_{600} had increased by 25%, 25% of the culture was withdrawn and replaced by an equal volume of fresh medium. At 100%, 200%, and 300% increase in the starting OD_{600}, 50% of the culture was withdrawn and replaced by an equal volume of fresh medium prewarmed to 37 C. All samples of cells were washed, shocked, and assayed for 5'-nucleotidase, acid hexose phosphatase, and cyclic phosphodiesterase. The results for 5'-nucleotidase are shown above. (A) Oleate to elaidate transition: (○) activity expressed by whole cells; (△) shock fluid. (B) Elaidate to oleate transition: (●) activity expressed by whole cells; (▲) shock fluid.*

there is a breakdown in the permeability barrier as shown by the facts that proteins are released and that the cells are generally more permeable to inhibitors such as actinomycin. Electron micrographs of unshocked cells of strain 30E show clear differences in cells grown in oleate and those grown in elaidate. Figure 3A shows a section through a culture supplemented with oleate, whereas Fig. 3B shows a culture supplemented with elaidate. Substitution of a *trans*-unsaturated fatty acid for the more normal *cis*-unsaturated fatty acid causes the outer membrane to extrude material similar to the outer membrane of the amino acid-starved auxotrophs observed by Knox et al. (16).

No differences were found in the lipopolysaccharide of the outer membrane as indicated by the fact that the outer membrane of oleate- and elaidate-supplemented cultures contained similar amounts of 2-keto-3-deoxyoctulosonic acid. Elaidate-supplemented cultures were slightly more sensitive to sodium dodecyl sulfate than were oleate ones. Elaidate cells lysed to 46% after 20 min at 37 C in 1 μg of SDS per ml compared to 31% lysis for oleate cells. Elaidate cells also showed only a slight inhibition of growth in the presence of sodium deoxycholate. In the presence of 0.2% and 0.5% detergent, the generation time of neither oleate cells (100 min) nor elaidate cells (175 min) was changed. In the presence of 1% sodium deoxycholate the oleate cells were still unaffected, whereas the elaidate cells showed a generation time of approximately 310 min, a value almost twice that of the control.

There was a marked difference in sensitivity to lysozyme action (Fig. 4). When either oleate or elaidate cells were washed with 0.154 M NaCl and suspended in 0.033 M Tris (*p*H 8) containing 20% sucrose, followed by the immediate addition of lysozyme, no lysis occurred; normal conversion to spheroplasts occurred with the addition of EDTA. However, if the cells were washed with 0.01 M Tris, *p*H 8, containing 0.03 M instead of 0.154 M NaCl, the elaidate cultures became sensitive to lysozyme in the absence of EDTA (Fig. 4A). Oleate cultures were much more resistant to lysozyme alone, and both types of cultures were converted to spheroplasts when EDTA was added immediately after the lysozyme (Fig. 4B). If the elaidate cells were allowed to remain in the Tris-sucrose solution for more than a few minutes, complete lysis could be effected by as little as 1 μg of lysozyme per ml in the absence of EDTA. This indicates that the outer membrane of cells grown in media supplemented with elaidate is porous enough for lysozyme to penetrate as far as the peptidoglycan layer. However, other permeability barriers appear to remain intact for strain 30E is insensitive to actinomycin in the absence of EDTA whether grown with oleate or elaidate supplementation.

DISCUSSION

The osmotic-shock technique has been useful in the identification of surface-localized enzymes, in the purification of these enzymes, and in the elucidation of the mechanism of

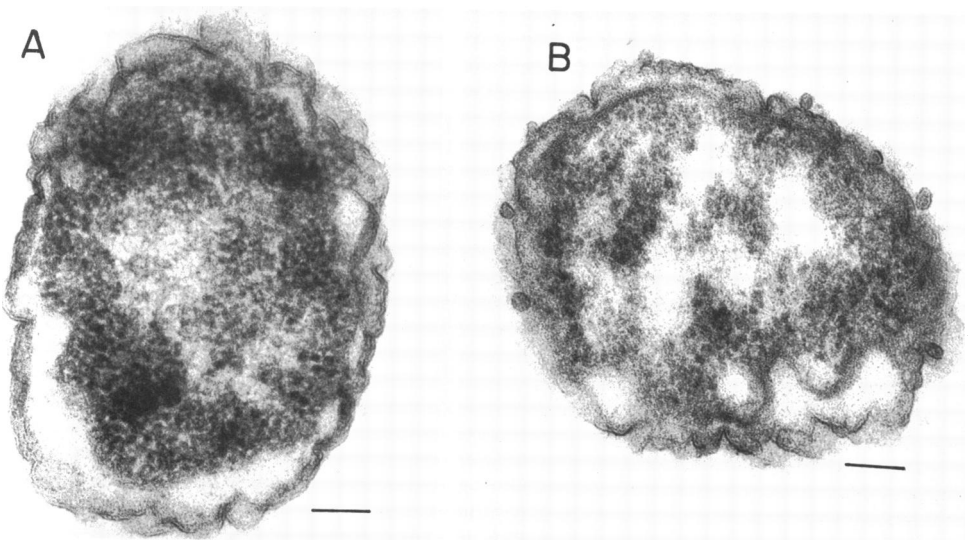

FIG. 3. *Electron micrographs of sections through cells from cultures of strain 30E supplemented with oleate (A) and elaidate (B). Cells were prepared for electron microscopy as described. Bar represents 0.1 μm.*

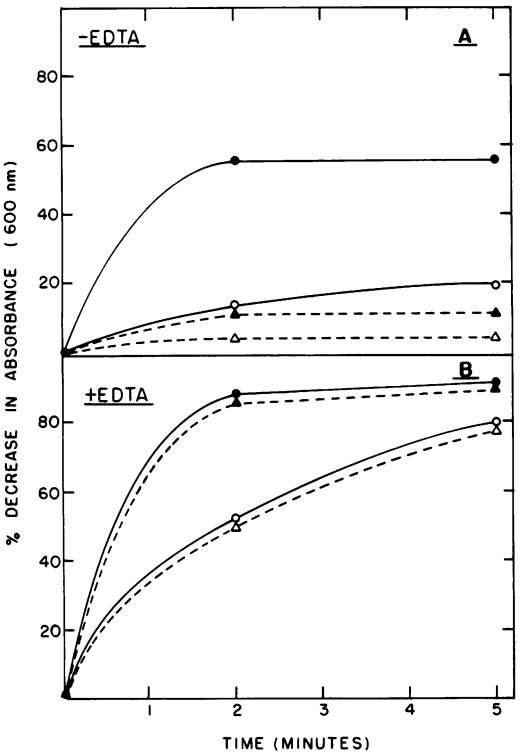

FIG. 4. *Sensitivity of strain 30E to lysozyme action. Cells were washed with 0.01 M Tris, pH 8, containing 0.03 M NaCl. Lysozyme was added and the decrease in absorbancy at 600 nm was followed as a function of time. (A) Absence of EDTA: (●) elaidate cells plus 10 μg of lysozyme per ml; (▲) oleate cells plus 10 μg of lysozyme per ml; (○) elaidate cells plus 5 μg of lysozyme per ml; (△) oleate cells plus 5 μg of lysozyme per ml. (B) Experiments performed with ethylenediaminetetraacetic acid added immediately after lysozyme: (●) elaidate cells plus 10 μg of lysozyme per ml; (▲) oleate cells plus 10 μg of lysozyme per ml; (○) elaidate cells plus 5 μg of lysozyme per ml; (△) oleate cells plus 5 μg of lysozyme per ml.*

active transport. However, the effectiveness of the shock procedure varies among different species of *Enterobacteriaceae*. *E. coli* cells release about 3 to 5% of their total cellular protein but release most of their surface proteins such as 3'-nucleotidase, 5'-nucleotidase, and certain transport proteins (21). *Proteus* and *Providentia* strains, on the other hand, release about 4% of their total protein but no 3'-nucleotidase or 5'-nucleotidase (21). These differences are thought to be due to differences in surface structure. It may be that the nucleotidases and acid hexose phosphatase are more firmly bound than most periplasmic proteins and are, therefore, subject to greater variations in release. Likewise, the conditions used for osmotically shocking exponential phase cultures of *E. coli* produce poor release of enzymes in stationary-phase cultures (22). These observations indicate that the composition of the complex cell surface plays a major role in the retention of periplasmic proteins and in their release by osmotic shock. Many of the lipid components of both inner and outer membranes contain unsaturated fatty acids. By using an unsaturated fatty acid auxotroph, we have investigated osmotic shock in cells containing either *cis*- or *trans*-unsaturated fatty acids.

The complexity of the cell surface in *E. coli* makes it difficult to localize the nature of the changes produced by growth in *trans*-unsaturated fatty acids. The inner membrane is definitely altered, as shown by alteration of temperature profile for transport reactions, both for sugars and (as shown here) for amino acids. Yet the maximal rates of transport of both arginine and glycine at temperatures above their transition temperature are very similar for cells containing either *cis*- or *trans*-unsaturated fatty acids in their membranes. This indicates that at temperatures above the transition temperature the ability of the amino acid-carrier complex to cross the membrane is unaffected by this change. Moreover, arginine and glycine transport appear to be affected similarly even though the presumed arginine carrier is shock releasable whereas the glycine carrier is not. The rigidity of elaidate-containing membranes at 23 C could prevent the release of certain periplasmic proteins. Since the same amount of protein is released from elaidate- and oleate-grown cultures, either the choice of the three enzymes studied was fortuitous in that the release of most other proteins is normal or else a new class of proteins is released from elaidate-grown cultures. However, one protein normally associated with the membrane, the Mg^{2+} (Ca^{2+}) adenosine triphosphatase, was found not to be released from osmotically shocked elaidate cells. Cerny and Teuber (5) found that cells treated with sublethal levels of the antibiotic polymyxin B release some surface proteins but that certain periplasmic enzymes such as 3'-nucleotidase and 5'nucleotidase are not released at the same time.

Lipid A of the outer membrane of *E. coli* also contains unsaturated fatty acids (4). Phospholipids also are important in stabilizing and maintaining the tertiary structure of the lipopolysaccharide and are necessary for the activity of the enzymes which form the lipopolysaccharide (24). Substitutions in phospholipid composition could cause the differences be-

tween oleate and elaidate cultures if *trans*-fatty acid-containing phospholipid destabilized the tertiary structure of the lipopolysaccharide. Gross changes in the outer membrane of elaidate-grown cells are visible in electron micrographs. More important is the observation that elaidate-grown cells are susceptible to lysozyme in the absence of EDTA, whereas oleate grown cells are not. This indicates that the peptidoglycan layer of the elaidate cells is exposed to lysozyme, suggesting that the arrangement of macromolecules is different in these cells. However, the similarity of the total amounts of 2-keto-3-deoxyoctulosonic acid in the lipopolysaccharide of elaidate and oleate cells indicates that alterations in the lipid A of the lipopolysaccharide have not prevented the attachment of the sugar components nor could the enzymatic activities of the lipopolysaccharide synthesizing enzymes be very different.

Very little elaidate is necessary to change the characteristics of an oleate membrane to those of an elaidate membrane (Fig. 2). Likewise, it takes a considerable amount of oleate to give an elaidate-derived culture the character of an oleate-derived culture. These events could arise if the elaidate were being randomly incorporated into the inner and outer membrane, spreading the effects of elaidate substitution generally. It is difficult to see how growth of inner and outer membrane at a single or limited number of sites could account for the data. This supports the hypothesis of Fox (31) that new membrane is formed at a large number of points along the cell surface.

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