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Murein and the Outer Penetration Barrier of *Escherichia coli* K-12, *Proteus mirabilis*, and *Pseudomonas aeruginosa*

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A penetration barrier operating outside the periplasmic enzyme penicillinase was studied in an ampicillin-resistant mutant of *Escherichia coli* K-12. Growth in the presence of lysozyme and sublethal concentrations of ampicillin partially opened the barrier. This could be recorded as an increased penetration of penicillin G, sodium cholate, and rifampin to their respective targets. Brief treatments with tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid and sodium cholate effectively impaired the barrier against penicillin and also caused leakage of penicillinase. Wild-type *E. coli* K-12, *Proteus mirabilis*, and *Pseudomonas aeruginosa* also showed an increased sensitivity to cholate after treatment with penicillins. Electron micrographs showed that lysis by cholate was due to a distortion of the cytoplasmic membrane causing a leakage of protein and RNA from the cells to the medium. Physiological data indicated that the increased sensitivity to cholate induced by growth in the presence of ampicillin or lysozyme was due to effects upon the murein. This was supported by measurement of the incorporation of ^{3}H-diaminopimelic acid. These results indicate that the murein sacculus either is a part of the penetration barrier or is responsible for holding the structure of the outer membrane together.

In gram-negative bacteria the outer region of the envelope (the outer membrane) constitutes a barrier against the penetration of different molecules into the cell. Smith (37) and Hamilton-Miller (14) have reported that penicillin treatment affects this barrier in *Escherichia coli* giving an increased penetration of penicillin into the cell. Leive et al. (18-20) reported that treatment with ethylenediaminetetraacetic acid (EDTA) makes the cells permeable to actinomycin D and also releases part of the envelope lipids and lipopolysaccharide. Normark (29, 30) has shown that a highly ampicillin-sensitive mutant of *E. coli* K-12 with a defect in the *envA* gene has lost part of the barrier function.

Strain D31 of *E. coli* K-12 was used in most of the experiments in the present study. It is a second-step, class III ampicillin-resistant mutant (4). This strain was selected for the present study because it contains an easily measurable penicillinase activity. In a previous study of strain D31 it was found that the initial rate of ampicillin hydrolysis was higher with buffer-suspended cells than with growing bacteria (8). However, after a lag period (see Fig. 1), the rate of hydrolysis obtained with growing bacteria approached what could be expected from studies of the purified penicillinase. It is here reported that this lag is due to difficulties in penicillin reaching the penicillinase which is known to be located in the periplasm (3). Lysozyme-EDTA spheroplasts of *E. coli* have been found to be more sensitive than whole cells to cholate lysis (28). In this paper the penetration barrier was studied by using increased sensitivity to cholate and rifampin as an indicator of damage to the outer layers of the cell envelope. The effect of penicillin on the penetrability properties of the outer envelope is not restricted to the mutant of *E. coli;* similar results were obtained with wild-type *E. coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Strains, media, and growth conditions. The main *E. coli* K-12 strain used was D31 which is resistant to 75 μg of D-ampicillin/ml (7). In some control experiments, its ancestor strain D11 was

included (4, 8). A few experiments were performed with *P. aeruginosa* Ps18S and *P. mirabilis* PM1 (12). The former strain is derived from strain 1822 (23) by the loss of the β-lactamase plasmid (38).

The complete medium used was LB of Bertani (2) but containing 0.2% glucose and supplemented with medium E (40) and vitamin B_1. It was solidified with 1.5% agar (LA plates). In some experiments the LB medium was supplemented with 20% sucrose (LB-S medium).

All experiments were performed at 37 C unless otherwise stated. The bacteria were grown as shaken cultures in a 37 C water bath. Growth was determined by measuring optical density in a Klett-Summerson colorimeter with filter W66. A reading of 100 Klett units corresponds to 4×10^8 log-phase cells per ml in LB medium.

Materials. DL-Ampicillin, D-ampicillin, and penicillin G were kindly donated by AB Astra, Södertälje, Sweden, and rifampin by I. Oeschger. Lysozyme was obtained from Sigma Chemical Co., St. Louis, Mo.; silica gel H was from Merck AG, Darmstadt, Germany; silicic acid from Mallinckrodt Chemical Works, New York; ^{32}P (orthophosphate, 50 Ci/mg of P) and ^3H-*meso*-α, ε-diaminopimelic acid (472 mCi/mmole) were from The Radiochemical Centre, Amersham, England. L-Leucine was from Sigma Chemical Co., and *meso*-α, ε-diaminopimelic acid from Calbiochem, Los Angeles, Calif.

Automatic iodometric assay for penicillinase. Novick's iodometric method, adapted to the Technicon Autoanalyzer was used (22). Penicillin G was used as substrate because it is hydrolyzed at a faster rate than is D-ampicillin by the penicillinase of strain D31 (7, 18).

Bioassay of ampicillin. The ampicillin assay was performed as described earlier by using *Sarcina lutea* as test organism (8).

Analysis of phospholipids. Labeling of phospholipid was performed for 10 generations in LB medium with ^{32}P-phosphate (1–2 μCi/ml) added. The cultures were always grown to a density of 4×10^8 cells/ml, and 5 ml of culture was precipitated with 2 ml of ice-cold 5% trichloroacetic acid. The precipitates were collected on glass filters (Whatman GF/C), washed five times with 0.12 N HCl containing 0.1 M KH_2PO_4 and extracted in 5 ml of chloroform-methanol (2:1, v/v) at 20 C overnight. The filters were removed, and the extracts were collected after filtration and concentrated to a volume of about 0.1 ml under a stream of nitrogen. The individual phospholipids were separated by thin-layer chromatography using as solvent system chloroform-methanol-acetic acid-water (25:15:4:1, v/v) as described by Skipsky et al. (35). The chromatograms were sprayed with ninhydrin and iodine vapors, and the phospholipids were identified with the aid of standards. The radioactive spots were scraped off into scintillation vials and counted in toluene scintillation liquid in a Nuclear-Chicago Mark I counter.

Fatty acids were esterified overnight at 65 C in methanol containing 2% H_2SO_4. The fatty acid methyl esters were extracted with petroleum ether and purified by silicic acid chromatography (11). The methyl esters were eluted with petroleum ether containing 2% ether and evaporated under a stream of nitrogen. The methyl esters were analyzed at 150 C in a Perkin-Elmer model 900 gas-liquid chromatograph, fitted to a column with 6% diethylene glycol succinate on Chromosorb W as stationary phase.

Assay of murein. Murein was measured by the incorporation of ^3H-diaminopimelic acid (Dpm) into trichloroacetic acid-precipitable material (36). However, a considerable incorporation into proteins takes place due to conversion of Dpm into lysine under these conditions. However, egg white-lysozyme treatment of acid-precipitated whole cells liberates most of the radioactivity incorporated into murein (34).

Strain D31 was grown in LB medium containing 584 μg of L-lysine and 20 μg of Dpm per ml. At a cell density of about 3×10^8 cells/ml, the cells were rapidly washed with 22 C LB-lysine medium to decrease the intracellular pool of Dpm and diluted five times into LB-lysine medium containing 4 μg of ^3H-Dpm per ml (2 μCi/ml). After two doublings, 5-ml samples were precipitated with 0.6 ml of 0 C 5% trichloroacetic acid and kept at 0 C for 15 min. The precipitates were then boiled for 15 min to remove the radioactivity incorporated into murein lipid intermediates and lysinyl-transfer ribonucleic acid (tRNA). The boiled precipitates were spun down (20,000 × *g*, 30 min), washed twice with ammonium acetate buffer (0.05 M, *p*H 7.6), and suspended in 5 ml of buffer containing lysozyme (500 μg/ml) by using sonic oscillation for a few seconds, and then digested overnight at 37 C. The samples were centrifuged (20,000 × *g*, 60 min), and the supernatant fluids were decanted into scintillation vials containing a glass fiber filter (Whatman GF/C). The filters were dried by evaporation, and the radioactivity was measured. About two-thirds of the radioactivity was liberated in these experiments.

Determination of the cell composition. Samples for determination of protein and RNA were pretreated by a modification (26) of the procedure described by Koch and Deppe (17). Protein was then assayed by the method of Lowry et al. (24) and RNA by the method of Fleck and Begg (13).

Electron microscopy. The cultures were immediately chilled in ice water and centrifuged. The pellets were fixed in ice-cold 4% (w/v) glutaraldehyde in 0.1 M phosphate buffer (*p*H 7.3) for 2 hr (32). They were rinsed in the same buffer and were postfixed for 1 hr at 4 C in 1% (w/v) OsO_4 dissolved in 0.1 M phosphate buffer (*p*H 7.3) containing 0.2 M sucrose. The cells were then washed for 1 hr in distilled water. Stepwise dehydration in ethanol was performed at room temperature, and embedding was performed in Epon 812 (25). The blocks were polymerized at 37 C overnight and for an additional period of 24 hr at 60 C. Sections were cut on an LKB Ultratome with glass knives. The sections were post-stained with uranyl acetate and lead citrate and were examined in a Philips EM 300 electron microscope.

RESULTS

Effects of pregrowth in the presence of

ampicillin and lysozyme. It was previously observed that the rate of ampicillin hydrolysis by growing cells of strain D31 was considerably faster than the rate of hydrolysis observed with bacteria suspended in phosphate buffer (8). This difference is illustrated in Fig. 1, which also shows that with growing cells there was a lag period before the hydrolysis started. This is not due to induction of penicillinase production (8).

It is known that, at low concentration, penicillin only acts on growing bacteria and that the penicillinase in strain D31 is entirely cell-bound and located in the periplasm (3). The difference between growing and nongrowing cells illustrated in Fig. 1 can therefore be interpreted as the result of a destruction of a penetration barrier taking place during the lag period and caused by growth with ampicillin. To test this interpretation, growing and nongrowing bacteria were pretreated in different ways. Afterwards, the substrate dependence of the cell-bound penicillinase was investigated. Figure 2 shows that untreated control cells were inefficient in hydrolyzing low concentrations of benzylpenicillin. Pregrowth in the presence of sublethal concentrations of ampicillin and to some extent also with lysozyme increased the rate of hydrolysis, particularly at low substrate concentrations. Most effective in this respect

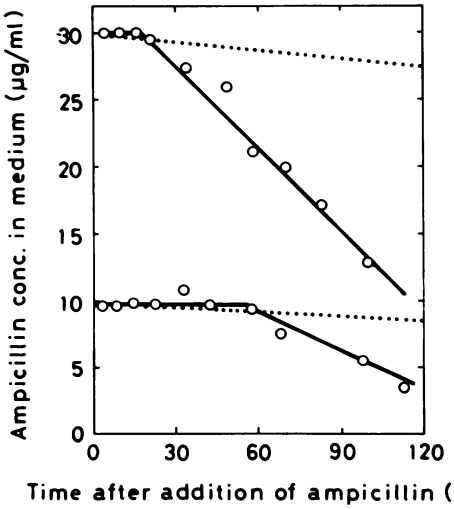

FIG. 1. *Hydrolysis of* DL-*ampicillin by cells of strain D31 growing exponentially in LB medium. At zero time (10⁸ cells/ml) ampicillin was added. The ampicillin concentration was determined by using a bioassay with Sarcina lutea as test organism. The dotted lines represent separate experiments on hydrolysis of ampicillin by buffer-suspended cells of strain D31 (from Burman et al., reference 8).*

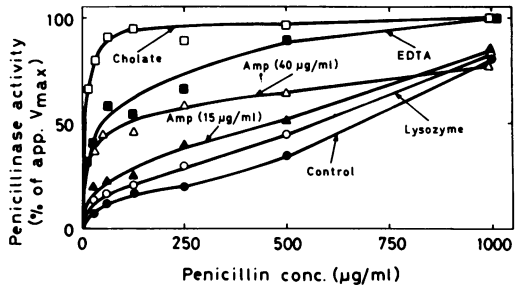

FIG. 2. *Hydrolysis of penicillin G by bacteria suspended in potassium-phosphate buffer (0.05 M, pH 7.4) at different substrate concentrations and after various pretreatments of the cells. Cells grown in LB medium (●); cells grown in LB medium plus 15 μg of ampicillin per ml (▲) and LB medium plus 40 μg of ampicillin per ml (△); cells grown in LB medium plus 20 μg or 100 μg of lysozyme per ml (○); cells briefly treated with tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (0.03 M, pH 8.0)-EDTA (5 mM (■) and with sodium cholate (10 mg/ml) (□). Pregrowth in the presence of ampicillin or lysozyme was performed for two doublings in LB medium. Brief treatments were performed at 20 C by suspending the cells in Tris-EDTA or sodium cholate, followed by immediate centrifugation.*

were cholate and EDTA, both of which released some of the cell-bound penicillinase.

If an early effect of ampicillin was the destruction of a barrier, growth with ampicillin could be expected to increase the sensitivity to cholate of the bacteria (28). This was confirmed in an experiment in which a growing culture of strain D31 was divided into two parts. To one was added D-ampicillin (40 μg/ml), and the other was kept as a control. At different times early, mid, and late log phase) cholate was added to both of the cultures. Figure 3 (left) shows that cholate slightly inhibited growth in the control culture, an effect which was more pronounced in the later part of the logarithmic growth phase. The right part of Fig. 3 shows that 30 min of growth with ampicillin increased cholate sensitivity so that immediate lysis was produced. The ampicillin concentration used (40 μg/ml) had no significant effect on the growth curve but caused filament formation owing to a block of cell division. The time required to make the cells highly cholate sensitive decreased with increasing concentration of ampicillin (Table 1).

A similar increase of cholate sensitivity was observed in the wild-type strain D11 after pregrowth with sublethal concentrations of ampicillin (0.5 and 1 μg/ml) (Fig. 4).

The experiments reported in Fig. 3 and Table 1 were repeated with 20% of sucrose in all

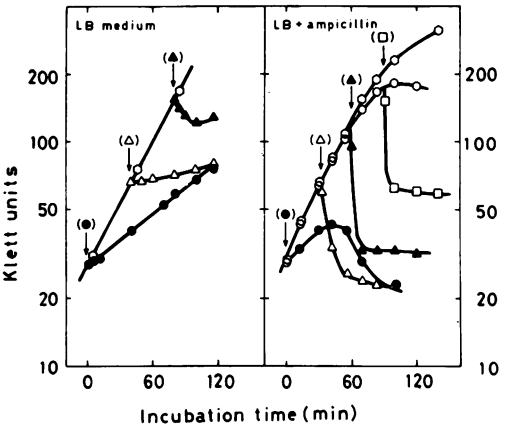

FIG. 3. *Effect of sodium cholate (3 mg/ml) added at different times (arrows) to strain D31 growing in LB medium in the absence or presence of* D*-ampicillin (40 μg/ml). The cells were pregrown in LB medium to a cell density of 5 × 10⁸ cells per ml. At zero time they were diluted five times into several flasks containing prewarmed LB medium with and without* D*-ampicillin. LB control (○); LB plus ampicillin control (○).*

TABLE 1. *Lag period before appearance of cholate sensitivity and start of ampicillin hydrolysis in exponentially growing cultures of E. coli D31 exposed to various concentrations of* D*-ampicillin*

D-Ampicillin concn (μg/ml)	Lag period (min)	
	Cholate sensitivity[a]	Ampicillin hydrolysis[b]
10	50–60	50
15	30–40	35
40	20–30	20

[a] The lag period was estimated from experiments analogous to those described in Fig. 3. The concentration of sodium cholate used was 3 mg/ml.

[b] Lag time before onset of ampicillin hydrolysis was estimated from Fig. 1 and analogous experiments (5).

media. This addition did not significantly change the result, although all responses were somewhat delayed due to the reduction of growth rate caused by the sucrose (from 30 min to 60 min doubling time). Hence, sucrose did not protect the cells from lysis by cholate.

Normark (29, 30) has reported a 100-fold increase in rifampin sensitivity of an *envA* mutant which has lost part of its barrier against antibiotics. The experiment reported in Fig. 5 shows that pregrowth of strain D31 with ampicillin (15 μg/ml) also increases rifampin sensitivity. There was virtually no killing by this concentration of ampicillin. The effect of ampicillin on rifampin sensitivity was of the same order of magnitude as that caused by the *envA* mutation.

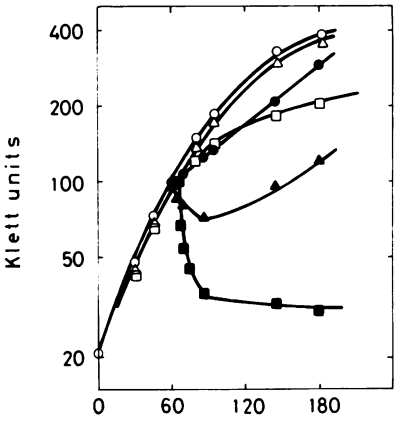

FIG. 4. *Effect of sodium cholate on the penicillin-sensitive E. coli strain D11 growing in LB medium in the presence of 0 (circles), 0.5 (triangles), or 1.0 (squares) μg of* D*-ampicillin per ml. At 100 Klett units, sodium cholate (5 mg/ml) was added (closed symbols).*

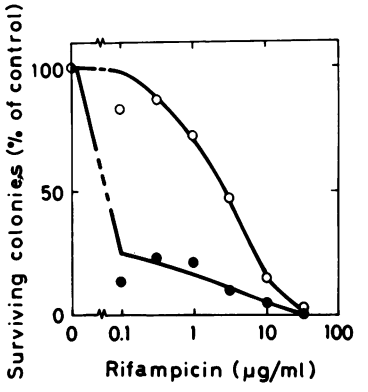

FIG. 5. *Effect of pregrowth with ampicillin on the sensitivity of E. coli strain D31 to rifampin (formerly known as rifampicin). The bacteria were grown in LB medium without or with* D*-ampicillin (15 μg/ml) for two doublings in the log phase and were then diluted into 0.9% (w/v) NaCl. Portions (0.1 ml) of the diluted cultures (about 400 cells) were spread on LA plates containing different concentrations of rifampin. Colonies were counted after incubation overnight. Control bacteria (○); bacteria pregrown with ampicillin (●). The colony count in the absence of rifampin was 4 × 10⁸ and 8 × 10⁷ for the control bacteria and the ampicillin-treated bacteria, respectively.*

The experiments reported above indicate that the penetration barrier against cholate was affected by treatment with ampicillin. To test

whether these low concentrations of ampicillin, which caused filament formation but not lysis of the cells, had affected the murein sacculus, we performed the experiment shown in Fig. 6. The cells were grown for two generations in the absence or presence of D-ampicillin (15 μg/ml), harvested, and treated with lysozyme and EDTA in the presence of 20% sucrose for 15 min. The sensitivity to sodium cholate was then measured. Both the control and ampicillin-treated cells were very sensitive to cholate after treatment with EDTA and lysozyme. The control cells were unaffected by cholate after EDTA alone, whereas the ampicillin-treated cells were cholate sensitive after treatment with EDTA in the absence of lysozyme.

Pregrowth with lysozyme increased the rate of hydrolysis of low concentrations of benzylpenicillin (Fig. 2). This indicates that lysozyme also affects the barrier. Therefore, a comparison was made between the cholate sensitivity of normal and lysozyme-treated bacteria. Figure 7 shows that the latter were more sensitive to cholate, an effect which was more pronounced at 100 μg of lysozyme per ml than at 20 μg per ml. Lysozyme did not have any effect on the growth rate.

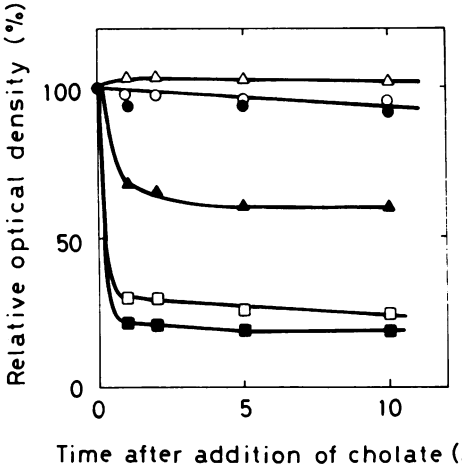

FIG. 6. *Effect of sodium cholate on ampicillin-treated cells of E. coli strain D31. The cells were pregrown in LB-S medium in two flasks. At a density of 10^8 cells/ml the culture was divided, and ampicillin (15 μg/ml) added to one part. Incubation was continued for two doublings. The cells were chilled in ice-water and harvested by centrifugation. Then they were resuspended in potassium phosphate buffer (0.05 M, pH 7.4) containing 20% sucrose (circles). In parallel experiments, 1 mM EDTA (pH 7.4) (triangles) or 1 mM EDTA plus lysozyme (20 μg/ml) (squares) was added. After 15 min at room temperature (zero time) 2 mg of sodium cholate was added per ml, and OD was recorded during another 10 min at room temperature. Open symbols refer to cells pregrown in the absence and closed symbols to cells pregrown in the presence of ampicillin. An OD of 100% refers in all cases to the time when lysozyme and EDTA were added.*

Effect of ampicillin and lysozyme on murein content of the bacteria. The effect of ampicillin and lysozyme on murein content of the bacteria was measured by incorporation of ^{3}H-Dpm as described in Materials and Methods. The incorporation was measured after two doublings in the presence of ampicillin or lysozyme (Table 2). The concentrations used did not decrease the growth rate as measured by optical density (OD). Treatment with ampicillin had some effect on cell morphology, but lysozyme did not change the appearance of the cells in the phase-contrast microscope (cf. Table 1). It is apparent from Table 2 that treatment with ampicillin as well as lysozyme slightly decreased the amount of murein in the cells.

Electron microscopy study of the cholate-induced lysis. The decrease in OD obtained by cholate (Fig. 3, 4, and 7) was found to be due to excretion of protein and RNA into the medium. Cells pregrown in the presence of 15 μg of D-ampicillin for two generations and then

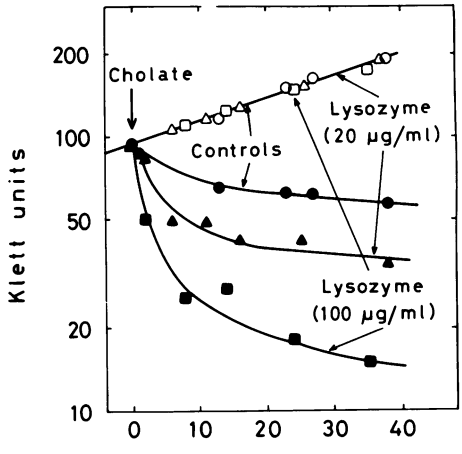

FIG. 7. *Effect of lysozyme on cholate sensitivity of E. coli strain D31. The bacteria were grown in LB medium to a density of 5 × 10^8 cells/ml, divided into six portions, and diluted five times into 37 C LB medium. Two cultures were kept as controls (circles), while 20 μg of lysozyme was added per ml to two cultures (triangles), and 100 μg of lysozyme was added per ml to the two last cultures (squares). After incubation for two doublings (zero time in the figure) cholate (3 mg/ml) was added to one series of flasks (closed symbols), and incubation was continued. Optical density was recorded.*

TABLE 2. *Effect of ampicillin and lysozyme on the incorporation of ^{3}H-Dpm into murein of E. coli strain D31*

Growth conditions (additions to LB medium)[a]	^{3}H-Dpm incorporated into murein (counts/min)[b]	Effect on cell morphology[c]
None	4,810	None
D-Ampicillin, 15 μg/ml	4,260	A slight tendency for filament formation
D-Ampicillin, 40 μg/ml	4,450	Filament formation
		Some swollen cells
Lysozyme, 200 μg/ml	3,660	None

[a] See Fig. 3 and 7.
[b] See Material and Methods.
[c] Studied in a Zeiss phase-contrast microscope.

treated with cholate (3 mg/ml) showed a 60% reduction in OD, and 57% of the protein and 59% of the RNA was found in the supernatant fluid.

Figures 8 through 10 are electron micrographs of strain D31 treated for two doublings by either D-ampicillin (15 μg/ml) or lysozyme (100 μg/ml). At a cell density of 100 Klett units, cholate (2 mg/ml) was added and the cells were harvested after an additional 15 min at 37 C. Figures 8a, 9a, and 10a are survey electron micrographs of control cells, ampicillin-treated cells, and lysozyme-treated cells, respectively. Figures 8b, 9b, and 10b show the same material at high magnification. It is apparent that addition of sodium cholate to cells pregrown in the presence of ampicillin or lysozyme caused a drastic decrease in the number of ribosomes of the cells. This corresponds to the fact mentioned above that RNA was found in the supernatant fluid. However, one important finding is also that the cells had not lost their general shape. The outer membrane and the cytoplasmic membrane form an almost continuous structure around the cells. At higher magnification it is clearly shown that the murein layer was still present in the ampicillin-treated cells (Fig. 9) whereas it was lost in the lysozyme-treated cells (Fig. 10). The latter result may, however, be an effect of the rather long treatment of the cells with cholate before harvest.

The outer membrane was intact in the cells treated with ampicillin or lysozyme, while the structure of the cytoplasmic membrane of these cells differed considerably from the control cells (Fig. 8–10). These pictures clearly show that cholate affected and distorted the cytoplasmic membrane.

Influence of growth phase on the penetration barrier. The results in Fig. 1 indicate that the penetration barrier was quite effective in exponentially growing bacteria and somewhat less effective in buffer-suspended cells. However, after growth in the presence of ampicillin the barrier was destroyed. In Fig. 1 and 2 these differences were demonstrated by the use of the cell-bound penicillinase. Parallel results were obtained by using cholate sensitivity as barrier indicator. Bacteria pregrown with ampicillin (40 μg/ml) were more susceptible to cholate lysis than were normally growing cells, and buffer-suspended cells showed an intermediate sensitivity.

Stationary-phase bacteria were found to be more resistant to cholate than exponentially growing cells. As much as 20 mg of cholate per ml was required for effective lysis of stationary-phase bacteria.

Phospholipid composition after treatment with ampicillin and lysozyme. Schnaitman (33) has recently shown that phospholipids are abundant in both the outer and the cytoplasmic membrane of *E. coli*. It was therefore investigated whether ampicillin or lysozyme treatment affected the barrier by changing the composition of the phospholipids. The results in Table 3 show that neither treatment caused any major alterations in either total phospholipids or the individual phospholipids obtained after separation by thin-layer chromatography. Analyses of the phospholipid fatty acids showed no major effects of ampicillin pregrowth (Table 4).

Effect of penicillins on cholate sensitivity of P. mirabilis PM1 and P. aeruginosa Ps18S. The experiment described in Fig. 3 and 4 was then repeated with *P. mirabilis* PM1 and *P. aeruginosa* Ps18S. As is shown in Fig. 11, 10 μg of D-ampicillin per ml had very little effect on the growth rate of *P. mirabilis* PM1. However, the ampicillin-treated cells were much more sensitive to cholate than the control cells. Analogous results were obtained with *P. aeruginosa* Ps18S treated with 400 μg of benzylpenicillin per ml (Fig. 12). In this case cholate was added at different times to different flasks allowing a study of the kinetics of the appearance of cholate sensitivity. The time required to get cholate sensitivity was 120 to 150, 90 to 120, and 60 to 90 min at 200, 400, and 600 μg of benzylpenicillin per ml, respectively (cf. Table 1 for *E. coli* strain D31).

DISCUSSION

Earlier studies (8) as well as the experiments in Fig. 1 show that cells of *E. coli* K-12

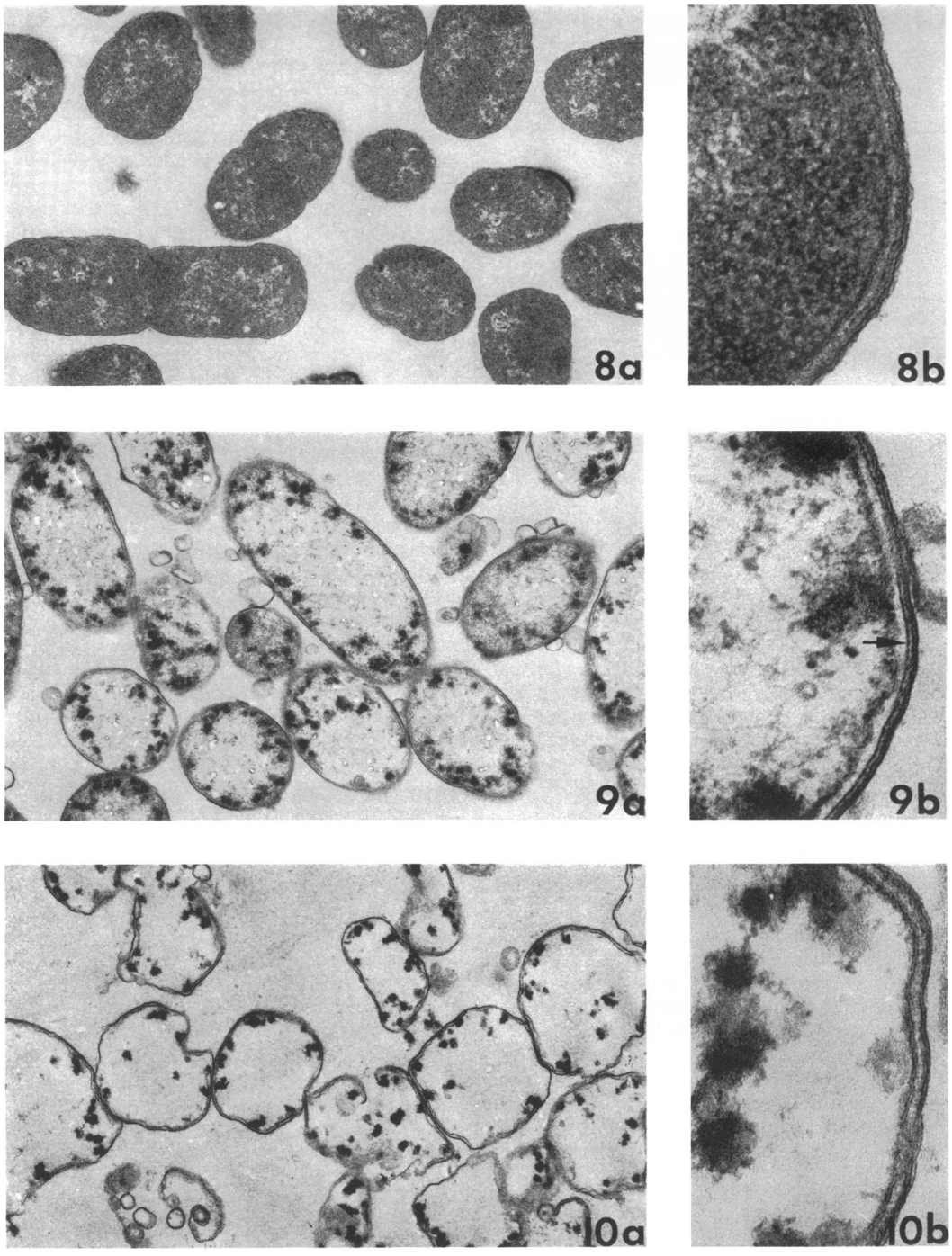

FIG. 8. *Electron micrograph of E. coli D31 grown in LB medium. Magnification: ×15,300 (a) and ×124,500 (b).*

FIG. 9. *Electron micrograph of E. coli D31 grown for two doublings in LB medium containing D-ampicillin (15 μg/ml) and then for another 20 min after the addition of sodium cholate (2 mg/ml) (cf. Fig. 3). Magnification: ×15,300 (a) and ×124,500 (b). In (b) the murein layer is indicated by an arrow.*

FIG. 10. *Electron micrograph of E. coli D31 grown for two doublings in LB medium containing lysozyme (100 μg/ml) and then for another 20 min after the addition of sodium cholate (2 mg/ml) (cf. Fig. 7). Magnification: ×15,300 (a) and ×124,500 (b).*

TABLE 3. *Effect of ampicillin and lysozyme on the phospholipids of E. coli D31*

Bacteria[a]	Phospholipids (%)[b]				Total phospholipid[c] (counts/min)
	DPG	PG	PE	Unknown	
Normal cells	2.9	15.1	73.6	8.2	11,906
Cells grown with D-ampicillin, 15 μg/ml	2.4	17.4	71.6	8.6	14,583
Cells grown with D-ampicillin, 40 μg/ml	2.6	17.8	71.1	8.4	10,077
Cells grown with lysozyme, 100 μg/ml	2.1	17.2	71.7	9.1	13,022

[a] Pregrowth in the respective medium was performed for two doublings in the logarithmic growth phase.

[b] The percentages are based on ^{32}P incorporation into the individual phospholipids. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE phosphatidylethanolamine.

[c] Total ^{32}P in chloroform-methanol (2:1, v/v) extracts from 2×10^9 log-phase cells labeled as described in Materials and Methods.

TABLE 4. *Effect of ampicillin on the composition of the total lipids of E. coli D31.*

D-Ampicillin concn (μg/ml)[a]	Fatty acids (%)[b]						
	C12:0	C14:0	C16:0	C16:1	C17:cy	C18:0	C18:1[c]
0	0.4	3.3	43.1	30.0	0.2	0.4	22.3
15	0.5	3.9	43.9	30.4	0.4	1.0	19.8
40	0.9	4.0	44.0	30.3	0.2	0.6	19.9

[a] The cells were grown in LB medium containing D-ampicillin for two doublings in the logarithmic growth phase.

[b] Number preceding colon indicates number of carbon atoms; number after colon designates degree of unsaturation. Cyclopropane fatty acids are designated by "cy."

[c] The main C18 fatty acid in *E. coli* is *cis*-vaccenic acid (16).

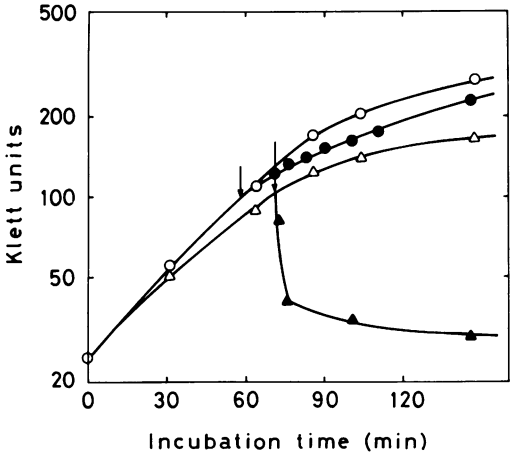

FIG. 11. *Effect of sodium cholate (10 mg/ml) on Proteus mirabilis PM1. The bacteria were grown in LB medium. At a cell density of 5 × 10⁸ cells per ml, the culture was diluted five times into flasks containing prewarmed LB medium (circles) or LB medium containing 10 μg of D-ampicillin per ml (triangles). Cholate (10 mg/ml) was added after two doublings at 37 C (closed symbols).*

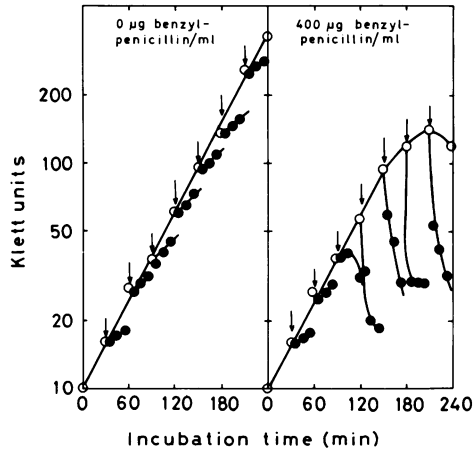

FIG. 12. *Effect of sodium cholate on Pseudomonas aeruginosa Ps18S. The bacteria were grown in LB medium to a cell density of 5 × 10⁸ cells per ml and diluted five times into several flasks containing 37 C LB medium (left) or LB medium containing 400 μg of benzylpenicillin per ml (right). At intervals cholate (15 mg/ml) was added to one flask in each series and incubation continued for another 25 min (closed symbols).*

hydrolyze penicillin poorly during active growth. However, the action of ampicillin on growing bacteria improves the ability of the cells to hydrolyze penicillins. The fact that the apparent V_{max} was not elevated by the treatments shows that there was no induction of penicillinase (see also reference 8). Growth in the presence of 40 μg of ampicillin per ml caused some decrease of the apparent V_{max}, probably because of leakage of enzyme. Pregrowth with ampicillin facilitates the penetration of low concentrations of penicillin through a barrier normally present (cf. 14, 37). Lysozyme had a similar but smaller effect on the bacteria.

Pregrowth in the presence of ampicillin made the cells more permeable not only to penicillin but also to rifampin and cholate (Fig. 2, 3, and 5). The target for ampicillin is outside the cytoplasmic membrane, whereas rifampin acts inside this structure. A synergistic effect with ampicillin is also observed for other antibiotics such as chloramphenicol, streptomycin, and polymyxin B, but not for tetracycline (Nordström and Burman, *unpublished data*). Synergism between penicillin and streptomycin (27, 41) has been shown to be due to altered uptake of streptomycin. Similar results were thus obtained with many functionally unrelated substances. This is a strong indication that there is an outer penetration barrier in *E. coli*. Furthermore, Table 1 shows that the time required for ampicillin to open the barrier was the same when cholate sensitivity or ampicillin hydrolysis was used as test parameter.

It should be stressed that the concentrations of ampicillin used were sublethal and did not decrease the growth rate (as measured by OD) during the time during which the penetration barrier was opened. Lysozyme had no effect on the growth of the cells (Fig. 3 and 7, Table 2).

The electron micrographs (Fig. 8–10) clearly show that cholate induces lysis by distorting the cytoplasmic membrane although there is no general breakdown of this structure—the outer membrane seems to be intact as judged from the electron micrographs. Analyses of RNA and protein confirmed that these substances leaked out from cells pregrown in the presence of ampicillin and then treated with cholate.

Since rifampin acts inside, cholate on, and ampicillin outside the cytoplasmic membrane, it follows that the penetration barrier must be located in the outer part of the bacterial cell envelope. The fact that lysis by cholate also took place in 20% sucrose makes it unlikely that osmotic forces are involved in this lysis.

That cholate sensitivity could also be induced by ampicillin pregrowth of the ampicillin-sensitive strain D11 indicates that a barrier function is present also in a wild-type strain. Even more important is the fact that analogous results were obtained with two other gram-negative bacteria, rather unrelated to *E. coli*, *P. mirabilis* (Fig. 11) and *P. aeruginosa* (Fig. 12). Hence, the barrier function seems to be quite similar in these three genera.

The damaging effects on the barrier obtained during growth with ampicillin and with lysozyme indicate that the murein is involved in the barrier function. Figure 6 and Table 2 indicate that the murein sacculus really was affected by these treatments. To our knowledge, this is the first demonstration that lysozyme is able to attack growing cells without cooperation with EDTA. It also shows that an intact murein is not a prerequisite for an osmotically stable cell. The outer regions also seem to be of importance for the stability of the cells. The various components of the cell envelope are kept together by ionic bonds, thus making the mechanical and osmotic stability of the cells dependent also upon structures other than the murein sacculus (1, 9, 10). Normark (30) has shown that cells with an impaired outer membrane are lysed when lysozyme is added to the growth medium.

The connection between the action of lysozyme and ampicillin and the function of the outer membrane may be as follows. Roberts et al. (31) have shown that growing *E. coli* in the presence of sublethal concentrations of penicillin reduced the degree of cross-linking in the murein. Höltje (Lunteren Symposium, 1971) has explained the decreased biosynthesis of murein in the presence of penicillin (cf. Table 2) by the penicillin sensitivity of the enzyme which provides end groups for the synthesis of new polysaccharide chains. It is well known that lysozyme cleaves the polysaccharide backbone of murein. Thus, these agents could make murein more "loose." An altered murein sacculus could affect the outer membrane through a special lipoprotein (7). The lipoprotein molecules are covalently linked to the Dpm residues of murein and have their lipid parts anchored in the outer membrane. If penicillin alters the arrangement of murein and lipoprotein, then the outer layers and the barrier function may also be disturbed. However, *Proteus mirabilis* and *Pseudomonas fluorescens* lack a lipoprotein which is covalently bound to the murein sacculus (6).

It is even possible that the murein sacculus itself functions as a barrier at least against cholate. Treatment with EDTA did not make

the cells cholate sensitive (Fig. 6), whereas after pregrowth in the presence of ampicillin or lysozyme the cells were lysed by low concentrations of cholate (Fig. 3, 4, 6, and 7). Furthermore, in gram-negative bacteria murein seems to be a tight structure that may act as a molecular sieve. According to Rehn (Lunteren Symposium, 1971) the average distance between two adjacent polysaccharide chains in the murein is 1.4 nm. The murein sacculus contains about 40% (w/w) (5) lipoprotein which presumably covers the outside of the murein backbone (Braun, Lunteren Symposium, 1971).

For an understanding of the molecular basis of the barrier, special attention must also be given to the lipopolysaccharide and the phospholipids. However, present data indicate that the biosynthesis of lipopolysaccharide is unaffected by penicillin (15). Therefore the phospholipids from strain D31 were studied before and after destruction of the barrier by ampicillin and lysozyme (Tables 3 and 4). The individual phospholipids showed a discrete increase of phosphatidylglycerol, and there was a small reduction of *cis*-vaccenic acid (C18:1) after ampicillin pregrowth.

The phospholipid and fatty acid composition may be of importance for the physical properties of the *E. coli* envelope. An increase in the amount of lipids in the cell envelope has been observed in *P. aeruginosa* treated with penicillins (39). The small effect on phospholipids caused by ampicillin may therefore be of relevance. However, the fact that lysozyme also affects the barrier would suggest that murein is essential for the function of the outer membrane as discussed above. This function could be a double one so that the barrier also prevents loss of periplasmic enzymes and metabolites.

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