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OUTER MEMBRANE OF *SALMONELLA TYPHIMURIUM* TRANSMEMBRANE DIFFUSION OF SOME HYDROPHOBIC SUBSTANCES*

HIROSHI NIKAIDO

Department of Bacteriology and Immunology, University of California, Berkeley, Calif. 94720 (U.S.A.)

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SUMMARY

The outer membrane, which is composed of lipopolysaccharide, phospholipids, and proteins, is a layer of the cell wall of Gram-negative bacteria, and apparently acts as a penetration barrier for various substances. It had been shown by other workers that "deep rough" mutants of *Salmonella typhimurium*, whose lipopolysaccharides lack most of the saccharide chains, were much more sensitive than the wild type strain to certain antibiotics and dyes, but not to others. We found that the former group of agents are usually hydrophobic and the latter group mostly hydrophilic. All hydrophilic antibiotics had molecular weights lower than 650, and one of them was shown to diffuse through the outer membrane at 0 °C. In contrast, some hydrophobic antibiotics had molecular weights in excess of 1200, and the rate of diffusion of one of them was shown to be extremely dependent both on temperature and on the structure of lipopolysaccharide present. These data and results presented elsewhere suggest, but do not necessarily prove, that most hydrophilic antibiotics diffuse through aqueous pores, whereas hydrophobic antibiotics and dyes mainly penetrate by dissolving into the hydrocarbon interior of the outer membrane. In contrast to the outer membrane of deep rough mutants, that of the wild type strain and less defective rough mutants was unusual among biological membranes in that it was practically impermeable to hydrophobic agents. It is proposed that the difference in hydrophobic permeability between the two types of strains is due to radical differences in the organization of the outer membrane, more specifically to the presence or absence of exposed phospholipid bilayer regions.

INTRODUCTION

Gram-negative bacteria are resistant to a number of antibiotics (e.g. erythromycin, some penicillins, rifamycin, and actinomycin D) and dyes (e.g. methylene blue and crystal violet) to which most Gram-positive bacteria are sensitive. Indeed, the dye

* This is paper VIII in the series on the outer membrane of bacteria. The preceding paper in this series is ref. 27.

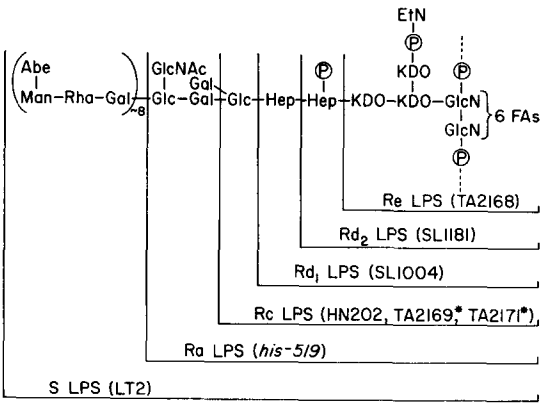

Fig. 1. Structure of lipopolysaccharides produced by *S. typhimurium* strains. The complete (S) or variously incomplete (Ra through Re) lipopolysaccharide molecules are produced by strains listed in parentheses (for review see ref. 2). Strains marked with asterisks are omp mutants, partially defective in the incorporation of proteins into the outer membrane [9]. Abbreviations: Abe, abequose; Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Glc, D-glucose; Hep, heptose; KDO, 2-keto-3-deoxyoctonic acid; EtN, ethanolamine; FA, fatty acid.

resistance of Gram-negative bacteria is the basis for such selective media as eosin-methylene blue agar. Since the cell wall of Gram-negative, but not of Gram-positive, bacteria contains an "outer membrane" layer [1], it has been often speculated that this outer membrane acts as a penetration barrier (see ref. 2 for review).

Outer membrane contains proteins, phospholipids, and lipopolysaccharide [2]. Mutants producing lipopolysaccharides with polysaccharide chains of different lengths are available in *Salmonella*, and provide a unique opportunity for examining how alterations in the structure of this integral component affect the postulated barrier properties of the outer membrane. Such studies, carried out by Roantree and coworkers [3] and by Schlecht and coworkers [4–6], yielded the following conclusions. Shortening of the saccharide chain in lipopolysaccharide has little effect on antibiotic and dye sensitivity until 80–90 % of the chain was lost, as in Rc mutants (Fig. 1). However, deletion of the next few sugar residues, as in Rd_1, Rd_2, or Re mutants (Fig. 1), produced cells with greatly increased sensitivity toward some antibiotics and dyes. In marked contrast, sensitivity of these "deep rough" mutants to other antibiotics was unaffected.

Although a large amount of data has been published on the changes in sensitivity, to my knowledge no explanation has been offered on why alterations of lipopolysaccharide structure produce such differential changes in the permeability of the outer membrane. In this study I shall show that the agents whose efficacy is affected by lipopolysaccharide structure are mostly hydrophobic, and appear to diffuse across the outer membrane by dissolution into the lipophilic interior of the membrane. It will also be proposed that the increased hydrophobic permeability in deep rough mutants is not a direct result of the alterations in lipopolysaccharide structure, but is due to an extensive change in the organization of the outer membrane. Most hydrophilic antibiotics, I shall propose, diffuse through water-filled pores, which are also utilized by oligosaccharides [7, 8].

MATERIALS AND METHODS

Bacterial strains. Derivatives of *Salmonella typhimurium* LT2 were used. The structure of lipopolysaccharides produced by these strains is shown in Fig. 1. The mutants called omp produce normal lipopolysaccharide but incorporate much less proteins into the outer membrane than the wild type does [9].

Chemicals. Antibiotics were obtained from the following sources: actinomycin D, chloramphenicol, chlortetracycline hydrochloride, D-cycloserine, neomycin sulfate, penicillin G potassium salt, polymyxin B sulfate, and tetracycline hydrochloride from Sigma; sodium salts of ampicillin (Polycillin-N®), methicillin (Staphcillin®), and oxacillin (Prostaphlin®) from Bristol; cephalothin sodium salt (Keflin®) and vancomycin hydrochloride (Vancocin®) from Eli Lilly; bacitracin sodium salt from Calbiochem; carbenicillin disodium salt (Geopen®) from Pfizer; novobiocin (Cathomycin®) from Merck, Sharp and Dohme; and rifamycin SV sodium salt from Schwarz-Mann. Nafcillin (sodium salt) was a gift from Dr. G. H. Warren, Wyeth Laboratories.

[^{14}C]Sucrose was obtained from Schwarz-Mann, and [^{3}H]dextran (16 500 daltons) was prepared by fractionating [*methoxy*-^{3}H]dextran from New England Nuclear Corp. [8].

Partition coefficients. Compounds were dissolved in 0.05 M sodium phosphate buffer, pH 7.0, and the aqueous solution was shaken at 24 °C with an equal volume of 1-octanol (Aldrich). After centrifugation, the concentration of the chemical in both aqueous and organic phases was determined, either by the ninhydrin reaction [10] or by spectrophotometry. When the latter method was used, values were corrected for the absorption of 1-octanol and for solvent-dependent differences of extinction coefficients.

Penetration of hydrophobic solutes. Cells were grown in L broth [11] (glucose omitted) at 37 °C with vigorous aeration by shaking, until the concentration reached about 0.4 mg dry weight/ml. Cells were collected by centrifugation for 5 min at $4000 \times g$, washed once with 0.1 M NaCl, and were resuspended in a minimal volume of 0.05 M sodium phosphate buffer, pH 7.0. Centrifugation and washing were done at room temperature, and the actual experiment was performed less than 1 h after the beginning of the harvesting operation. The cell suspension (2.1 ml containing about 1 g wet weight of cells) in a test tube was incubated for a few minutes in a water bath at a given temperature and then 0.3 ml of an antibiotic solution (50 mg/ml nafcillin or 4 mg/ml rifamycin SV in 0.05 M sodium phosphate, pH 7.0) was added with mixing. Portions (about 0.3 ml) of the suspension were taken out at intervals and were immediately transferred to well-chilled heavy-walled glass centrifuge tubes in an ice bath. The rapid cooling of the suspension usually stopped the diffusion process (see Results). The samples were then centrifuged at $25\,000 \times g$ for 10 min at 0 °C, and the antibiotic concentration in the supernatants was determined spectrophotometrically after proper dilution in water.

If entry and exit of solute are simple diffusion processes, then the rate of change in external solute concentration can be expressed as follows.

$$-\frac{dC_{ex}}{dt} = -\frac{1}{V_{ex}} \cdot \frac{dS_{ex}}{dt} = \frac{1}{V_{ex}} (kC_{ex} - kC_{in}) \qquad (1)$$

Here C, S, V, and t correspond to solute concentration, amount of solute, volume of compartment, and time, respectively, and subscripts ex and in refer to the extracellular compartment (i.e. medium) and intracellular compartment, respectively. If we consider that $C_{ex} \cdot V_{ex} + C_{in} \cdot V_{in} = S$, and assume that at $t = \infty$, $C_{in} = C_{ex} = C^{\infty}$, Eqn. 1 becomes

$$-\frac{dC_{ex}}{dt} = k \frac{V_{in}+V_{ex}}{V_{in} \cdot V_{ex}} (C_{ex}-C^{\infty}).$$

Integration gives

$$\ln(C_{ex}-C^{\infty}) = \ln(C_{ex}^{0}-C^{\infty}) - \frac{k(V_{in}+V_{ex})}{V_{in} \cdot V_{ex}} t \qquad (2)$$

where C_{ex}^{0} is the initial concentration of the solute in the medium. Since bacterial cells are surrounded by a rigid peptidoglycan layer, V_{in} and V_{ex} will remain practically constant for the duration of a given experiment. Eqn. 2 can therefore be rewritten as

$$\log(C_{ex}-C^{\infty}) = \log(C_{ex}^{0}-C^{\infty}) - k't \qquad (3)$$

by introducing a new constant, k'. Thus if a solute is diffusing into the cell according to Eqn. 1, a plot of log $(C_{ex}-C^{\infty})$ versus time should give a straight line, as indicated by Eqn. 3.

Two controls were usually run: firstly, cell suspension cooled to 0 °C prior to the addition of the antibiotic solution (tube A); and secondly antibiotic solution added to 0.05 M sodium phosphate, pH 7.0, with no cells (tube B). The wet weight of the cells in 1 ml of incubation mixture was also determined after centrifugation. Since 1 g wet weight of cells had a cellular volume of 0.51 ml as determined by the use of [^{3}H] dextran (for methods see ref. 8), cell volume in 1 ml of the incubation mixture was calculated from the measured wet weight. The expected $C_{ex}{}^{0}$ is (concentration in tube B)/(1−cell volume). If the antibiotic does not penetrate at 0 °C and if it does not adsorb to cellular components, then the $C_{ex}{}^{0}$ for tube A should agree with the expected value. This was found to be the case (see Results). The expected C^{∞} was calculated as (concentration in tube B)/(1−cell volume $\times$0.2), on the assumption that about 20 % of the cell volume is occupied by macromolecules impenetrable to the antibiotic. This expected C^{∞} value usually agreed with the C_{ex} values obtained after long incubation with permeable (e.g. Re-type) cells, indicating the absence of adsorption, or accumulation by active transport, or degradation of the compounds by the cells.

Penetration into spheroplasts. EDTA-lysozyme spheroplasts were prepared by a modification of the procedure of Birdsell and Cota-Robles [12]. Strain LT2 was grown in 4.5 l of L broth with vigorous shaking at 37 °C, and the cells were harvested rapidly by centrifugation (6000 $\times g$ for 5 min at 4 °C) when the density of the culture reached 0.2 mg dry weight/ml. The inside walls of the centrifuge tubes were carefully wiped with tissue paper, and the cells were resuspended in 95 ml of ice-cold 0.75 M sucrose/0.01 M Tris−HCl buffer, pH 7.5. 5 ml of the sucrose/Tris buffer containing 10 mg of lysozyme (Sigma) and 10 mg of pancreatic deoxyribonuclease (Sigma, type I) were then added. After 2 min at 0 °C, the suspension was diluted slowly by adding, over a period of 5 min, 200 ml of ice-cold water with constant and gentle stirring. The suspension was then centrifuged at 3500 rev./min for 10 min at 4 °C in a GS-3 rotor of

a Sorvall RC-2B centrifuge. The supernatant solution was decanted, and the pellet was carefully resuspended with 1.5 ml of 0.25 M sucrose/0.01 M Tris—HCl buffer, pH 7.5, containing 1 mg each of pancreatic deoxyribonuclease and ribonuclease (Sigma, type XIIB). The conversion of these lysozyme-treated cells into spheroplasts was done in the reaction mixture, in order to avoid the lysis of spheroplasts due to manipulations such as centrifugation or resuspension. The suspension mentioned above (2.0 ml) was added to a test tube which was kept in a water bath at 14 °C. After a few minutes of temperature equilibration, 0.1 ml of 0.25 M sucrose containing 0.05 M EDTA was added. Microscopic observation in preliminary experiments showed that the addition of EDTA converted more than 90 % of the cells into spherical forms within 1 min. 1 min after the addition of EDTA, 0.3 ml of 0.25 M sucrose containing 50 mg/ml nafcillin was added, and the time course of diffusion of nafcillin into spheroplasts was followed as described in the preceding section, except that the centrifugation was done at 2500 rev./min for 15 min at 0 °C in a SS-34 rotor of a Sorvall RC-2B centrifuge.

Penetration into plasmolyzed cells. This was carried out essentially as described earlier [8]. Briefly, a thick suspension of cells was incubated with [^{3}H]dextran and [^{14}C]sucrose in the presence of a plasmolyzing agent, e.g. 0.4 M sucrose, and then were centrifuged. Determination of ^{3}H and ^{14}C concentrations both in the supernatant and pellet allowed us to calculate spaces (in the pellet) that are permeable to dextran and sucrose. With unplasmolyzed cells, the two spaces were almost equal. With plasmolyzed cells, however, the sucrose-permeable space was significantly larger than the dextran-permeable space, as sucrose, but not dextran, penetrated into the periplasmic space enlarged by plasmolysis.

Here these experiments were performed in the presence of carbenicillin or nafcillin. In this case, the antibiotic-impermeable space (v) was first calculated from the antibiotic concentration in the supernatant (C_{ex}) and that in the control tube without cells (C_{cont}) by the equation $C_{ex} = C_{cont} \cdot V_{tot}/(V_{tot} - v)$, where V_{tot} refers to the total volume of the incubation mixture. The antibiotic-permeable space was then calculated by subtracting impermeable space from the pellet volume, which was calculated from the pellet weight. The antibiotic concentrations were determined spectrophotometrically after dilution in water (for carbenicillin, at 220 nm after 100-fold dilution; for nafcillin, at 227 nm after 500-fold dilution). Correction was made for the absorbance due to the leakage of cellular material, by running control tubes without the antibiotic; this correction never exceeded 10 % of the absorbance of the antibiotic.

RESULTS

Inhibitors that are more effective on deep rough mutants are usually hydrophobic

I suspected that the increased sensitivity of deep rough mutants to certain antibiotics and dyes might be related to the hydrophobicity of the inhibitors, and that hydrophobic molecules might diffuse through outer membrane by a mechanism entirely different from that utilized by hydrophilic molecules. As an index of hydrophobicity, partition coefficients of various reagents were determined in 1-octanol/0.05 M sodium phosphate buffer, pH 7.0. The buffer, rather than water, was used to eliminate any effect of counter ions present in antibiotic preparations. There indeed

TABLE I

HYDROPHOBICITY, SIZE, AND RELATIVE EFFICACY AGAINST DEEP ROUGH MUTANTS, OF VARIOUS INHIBITORY AGENTS

Group I agents are about equally active against deep rough mutants and their parent smooth strains of *Salmonella*. In contrast, group II agents are much more inhibitory to deep rough mutants than to the wild type strains. Efficacy ratio values were calculated from the data of Roantree and coworkers (ref. 3; personal communication) in A and from those of Schlecht and coworkers [4–6] in B. The numbers correspond to the ratios (S/Re) of minimum inhibitory concentrations (in A) or of the lengths of growth zones on gradient plates (in B). Partition was determined in the 1-octanol/0.05 M sodium phosphate, pH 7.0, system at 24 °C. Neomycin, cycloserine, and polymyxin concentrations were measured with ninhydrin. Concentrations of the other agents were determined spectrophotometrically, either at the wavelength of an absorption peak or at 220 nm. The initial concentration of the agents in buffer was usually less than 1 mg/ml.

Group	Agent	Efficacy ratio (deep rough/wild type)		Partition coefficient	Molecular weight
		A	B		
I	Chloramphenicol		1.0	12.4	323
	Chlortetracycline		0.8	0.31	479
	Tetracycline	0.5	0.5	0.07	444
	Peniclilin G	1.0		0.02	334
	Methicillin	2		0.01	379
	Ampicillin	1.0		< 0.01	349
	Cephalothin	0.5		< 0.01	395
	Carbenicillin		1.0	< 0.01	378
	Neomycin	1.0		< 0.01	615
	Cycloserine		0.7	< 0.01	102
II	Actinomycin D		> 10	> 20	1255
	Novobiocin	> 20		> 20	613
	Phenol		> 10	> 20	94
	Crystal violet		> 10	14.4	408
	Rifamycin SV		> 10	8.8	698
	Malachite green		> 10	4.2	365
	Nafcillin	10		0.31	414
	Bacitracin	5		0.12	1411
	Oxacillin		10	0.07	418
	Polymyxin B	3		< 0.05	≈ 1200
	Vancomycin	10		< 0.01	≈ 3300

was a clear correlation between the hydrophobicity of inhibitors and their relative effectiveness against deep rough mutants (Table I).

Most agents whose effectiveness was not increased by mutational alteration of lipopolysaccharide (called "group I agents" hereafter) were hydrophilic in the sense that their partition coefficients were lower than 0.02. (The exceptions will be considered in Discussion). These substances presumably diffuse through water-filled "pores" in the outer membrane (see Discussion), as do oligosaccharides [7, 8].

In contrast to the group I agents, almost all of the group II agents, which are much more effective against deep rough mutants than against wild type cells, were hydrophobic with partition coefficients higher than 0.07 (Table I). (The exceptions

will be considered in Discussion). The simplest explanation seems to be that these hydrophobic molecules penetrate through the outer membrane mainly by dissolving into the hydrocarbon interior of the membrane (see below and Discussion).

Diffusion of a hydrophobic substance across outer membrane

The increased sensitivity of deep rough mutants to hydrophobic inhibitors only suggests an increased rate of penetration of such inhibitors across the outer membrane. A pioneering attempt to measure the actual rates was done by Gustafsson and coworkers [13] by using gentian violet, but the dye was heavily adsorbed to cellular constituents. We therefore used nafcillin, which showed only negligible adsorption to cellular constituents, and measured the rate of diffusion of this compound across the cell envelope into the cytoplasm (Materials and Methods).

An example of the diffusion experiment with an Re strain is shown in Fig. 2. It should be noted that both the 0 and the 5-min concentrations measured were quite close to those predicted on the assumption that nafcillin existed only in extracellular space at 0 min and attained equal concentration in extra- and intracellular spaces by 5 min (see Materials and Methods). Furthermore, when the data were replotted in the manner described in Materials and Methods, a straight line was obtained (inset, Fig. 2), as predicted by the simple diffusion model.

In similar experiments with a series of lipopolysaccharide mutants at 22 °C, it was found that S, Ra, and Rc strains were essentially impermeable to nafcillin, whereas "deep rough" (Rd_1, Rd_2, and Re) mutants allowed a rapid penetration (Table II). (Although the constant k' is theoretically dependent on V_{in} and V_{ex} (Eqn. 2 in Materials and Methods), the values of k' do reflect relative rates of permeation as

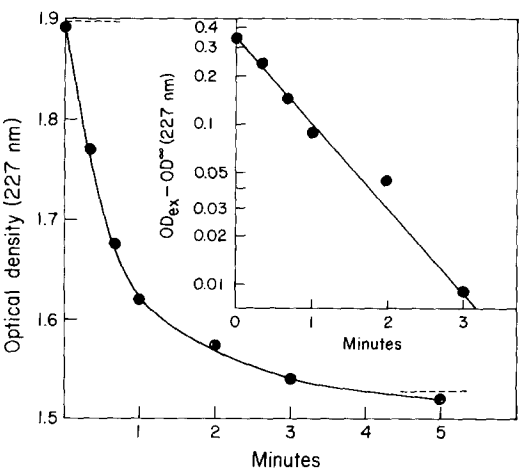

Fig. 2. Diffusion of nafcillin into TA2168 (Re) cells at 22 °C. Nafcillin was added to a thick suspension of cells and portions were chilled at various times and centrifuged (Materials and Methods). Nafcillin concentration in the supernatant solution was determined spectrophotometrically at 227 nm after 500-fold dilution in water. The dotted lines correspond to the absorbances for expected initial (C_{ex}^{0}) and final (C^{∞}) concentrations of nafcillin, calculated as described in Materials and Methods. Inset shows the replot of the data, in a form expected to yield a straight line (see Materials and Methods).

TABLE II

RATES OF DIFFUSION OF NAFCILLIN INTO VARIOUS STRAINS OF *S. TYPHIMURIUM* AT 22 °C

Lipopolysaccharide type produced by the strain*	Rate constant** (min^{-1})
S	0.005–0.007 (3)
Ra	0.005–0.007 (4)
Rc	0.005–0.009 (4)
Rd_1	0.1–0.6 (4)
Rd_2	0.1–0.5 (3)
Re	0.6–1.8 (6)
Rc (omp)	0.2–1.5*** (6)

* See Fig. 1 for lipopolysaccharide structure and for strains used.

** The numbers listed correspond to the pseudo first-order rate constant, k', in Eqn. 3, obtained under the conditions described in Materials and Methods. Numbers of experiments are listed in parentheses.

*** The penetration into omp mutants first proceeded with these rates, but usually became slower after a few minutes, for unknown reasons.

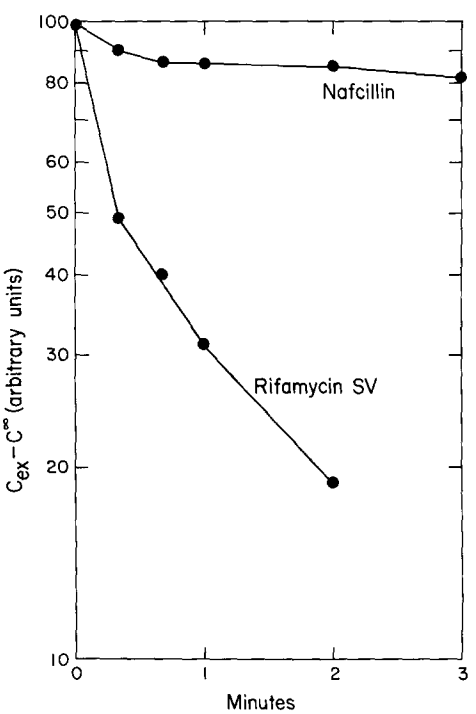

Fig. 3. Diffusion of nafcillin and rifamycin SV into TA2168 (Re) cells at 15 °C. Rifamycin SV was determined spectrophotometrically at 450 nm. For other conditions see Materials and Methods and the legend to Fig. 2. The non-linearity of the rifamycin SV curve might be due to the fact the agent continues to penetrate slowly even at low temperatures; the resulting delay in the arrest of diffusion process will affect the earlier time points more seriously.

similar amounts of cells were used in all experiments.) Thus it was clear that the rate of penetration of a hydrophobic substance was affected by alterations in lipopolysaccharide structure.

Interestingly, the omp mutants with reduced amounts of proteins in the outer membrane [9] allowed a rapid initial diffusion of nafcillin, in spite of the presence of the normal amounts of Rc-type lipopolysaccharide (Table II); this result will be considered in Discussion.

Effect of hydrophobicity on diffusion rate

A number of group II agents were compared as to their rates of penetration through the outer membrane. Only two of these substances were convenient to assay and did not adsorb to cellular constituents. Nevertheless, the more hydrophobic compound, rifamycin SV, penetrated much faster than nafcillin (Fig. 3).

Effect of temperature on the diffusion rate

The penetration of nafcillin was extremely temperature dependent (Fig. 4). The data shown in Fig. 4 can be interpreted in two ways. (a) There could be a sudden, discontinuous increase in permeability in the range 14–22 °C, which could correspond to the transition temperature of the lipids in the outer membrane. (b) Alternatively, the penetration process could have high temperature coefficients throughout a much wider temperature range, and the apparently sudden increase in the 14–22 °C range

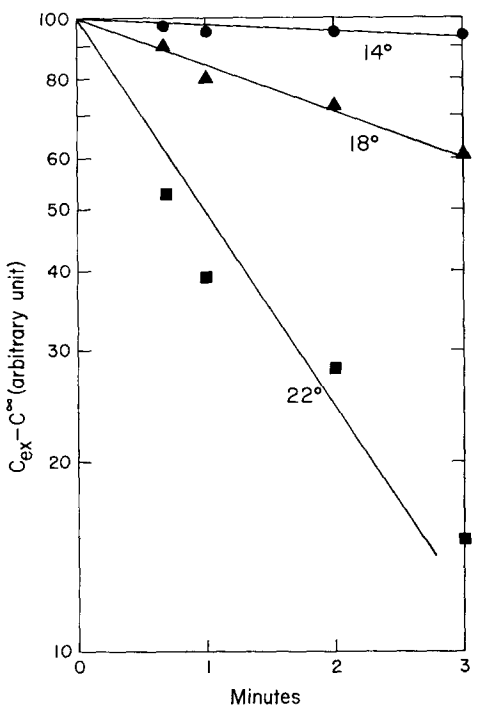

Fig. 4. Diffusion of nafcillin into TA2168 (Re) cells at different temperatures. For details see Materials and Methods and the legend to Fig. 2.

could have been caused entirely by the technical reason that the penetration rates happened to fall into the easily measurable range at these temperatures. According to the hypothesis a, a similar increase in permeability should be seen in the same temperature range with all hydrophobic antibiotics. Experiments with rifamycin SV showed, however, that the rate changes roughly corresponding to those shown in Fig. 4 took place in the 8–16 °C range (not shown). These results support the interpretation b, and suggest that with the more rapidly penetrating rifamycin SV the rates came into the measurable range earlier, or at a lower temperature. We thus conclude that "transitions" are technical artefacts and that the temperature coefficients of the penetration remains high over a wide temperature range.

Diffusion through the cytoplasmic membrane

In experiments described so far, the agents had to diffuse through both the outer and inner membranes to reach the cytoplasm. In order to find out whether the cytoplasmic membrane acted as a significant penetration barrier for nafcillin, we used EDTA-lysozyme spheroplasts, in which the cytoplasm was known to be surrounded by a continuous layer of the cytoplasmic membrane, with fragmented and curled-up remnants of outer membrane attached to it [12]. When the rate of penetration of nafcillin into spheroplasts of strain LT2 was measured at 14 °C as described in Materials and Methods, it was found to be extremely rapid: even at the earliest time point (20 s), we found that the $(C_{ex} - C^{\infty})$ value was already down to about 20 % of the $(C_{ex}{}^{0} - C^{\infty})$ value. In this experiment, 1 ml of the reaction mixture contained 0.4 g wet weight of spheroplasts, an amount comparable to the amount of cells used in experiments with intact cells (0.42 g/ml). The pseudo first-order rate constants are thus roughly comparable, if we disregard changes in cell surface area that must accompany the formation of spheroplasts. At 14 °C, the k' value with spheroplasts is certainly higher than 1 min^{-1}. This is orders of magnitude higher than the rate of penetration into intact cells, even of the Re type, at this temperature (Fig. 4). We therefore conclude that in intact cells the rate-limiting barrier against the diffusion of nafcillin corresponds to the outer membrane, even in strains possessing the most permeable outer membrane.

In order to find out whether spheroplasts have damaged cytoplasmic membranes, [^{3}H]dextran was added to the incubation mixture in some experiments. The apparent intercellular space, or dextran-permeable space, in the pellet, calculated on the basis of the ^{3}H concentrations in the pellet and the supernatant (see ref. 8), was 50–59 % of the pellet volume. These values are quite similar to the values of intercellular space obtained in the pellets of intact cells (49 %), and suggest that most spheroplasts still retain intact penetration barriers against dextran.

Diffusion of carbenicillin into plasmolyzed cells

It was proposed earlier that most group I agents penetrate through water-filled pores in the outer membrane. If so, the diffusion of these agents should occur rapidly even at low temperatures. This prediction was tested by determining the diffusion of one group I agent, carbenicillin, into HN202 (Rc) cells that had been plasmolyzed in 0.4 M sucrose (Materials and Methods). When the cells were centrifuged after 10 min contact with carbenicillin at 0 °C, a pellet with a volume of 201 μl was obtained. [^{3}H]Dextran distributed in 99 μl of space in the pellet, a space corresponding to the

intercellular space. [^{14}C]Sucrose, however, distributed in 146 μl of space, which presumably corresponds to the sum of the intercellular space and the periplasmic space. From the carbenicillin concentration in the supernatant, we could show that it distributed in 140 μl of the space within the pellet, and we conclude that the drug penetrates fully through the outer membrane at 0 °C and enters the expanded periplasmic space, but does not diffuse across the cytoplasmic membrane at this temperature.

In a control experiment, nafcillin was added to similar batches of plasmolyzed cells of HN202 and TA2168 (Re) at 0 °C. As expected, the space in which nafcillin distributed was approximately equal to the intercellular, or dextran-permeable, space, confirming that nafcillin does not penetrate through the outer membrane at 0 °C.

DISCUSSION

It has been known, since 1969, that deep rough mutants of *Salmonella* [3–6] and *Escherichia coli* [15, 16] are unusually sensitive to certain dyes and antibiotics, but not to others. The mechanism underlying this alteration of sensitivity, however, has remained obscure. I have shown in this paper that the agents which are more active toward deep rough mutants are usually hydrophobic, whereas those whose efficacy is not altered by lipopolysaccharide structure are mostly hydrophilic (Table I). These results suggested that the two groups of agents penetrate outer membrane by different mechanisms.

We have recently studied the mechanism of diffusion of oligosaccharides across the outer membrane of *Salmonella* [7, 8]. This pathway of diffusion is characterized by a sharp exclusion limit in terms of molecular size [7, 8]. Our recent reconstitution studies showed that protein aggregates containing only three species of outer membrane proteins could combine with phospholipids and lipopolysaccharide, and produce membrane vesicles that are permeable to various sugars, sugar phosphates, amino acids, nucleotides, and polyethylene glycol (Nakae, T., J. Biol. Chem., in the press, see ref. 36). Since facilitated diffusion of these diverse compounds would obviously require many different kinds of carrier proteins, these results strongly support the presence of aqueous pores.

Although we have not studied the diffusion of hydrophilic, group I antibiotics in detail, it seems reasonable to assume that they diffuse through aqueous pores, especially in view of the lack of specificity in this mechanism, as described above. Available results on the penetration of group I agents are all at least consistent with this hypothesis. (i) The exclusion limit of the pores in *S. typhimurium* is between 600 and 800 daltons, when determined by the use of oligosaccharides [7, 8] (Nakae, T., J. Biol. Chem., in the press, see ref. 36). All of the group I agents had molecular weights less than 650 (Table I). (ii) Since the pores are presumably formed by proteins [17] (Nakae, T., J. Biol. Chem., in the press, see ref. 36), this diffusion mechanism should not be affected drastically by alterations of lipopolysaccharide. This indeed appeared to be the case for the diffusion of antibiotics (Table I), as has been shown for oligosaccharides as well [8]. (iii) Since the penetrating molecules always diffuse in water, the temperature dependence of the penetration process should be equivalent to that of simple diffusion in water [14]. We found that the diffusion of carbenicillin into the periplasmic space takes place even at 0 °C (Results).

The penetration mechanism for group II agents seems to have properties very different from those of the "hydrophilic pathway". Thus the mechanism (i) seems to have no clear-cut size limit (Table I), (ii) is profoundly affected by the structure of lipopolysaccharide present in the outer membrane (Table II), and by the amount of proteins in the outer membrane (see results on omp mutants in Table II), and (iii) is characterized by extremely high temperature coefficients (Fig. 4). These results do not necessarily rule out facilitated diffusion as the underlying mechanism. However, in view of the observation that most of the group II agents are hydrophobic (Table I), and that outer membrane contains less than a dozen species of proteins [18], the simplest hypothesis appears to be that most group II agents penetrate through the outer membrane mainly by dissolving into the hydrocarbon interior of the membrane and then diffusing into the aqueous phase on the other side of the membrane. This is of course the classical mechanism that has been assumed for the penetration of various hydrophobic compounds through biological membranes [19], and a high temperature coefficient is one of the diagnostic properties of this mechanism [14]. Our observation that the more hydrophobic rifamycin SV penetrated faster than nafcillin (Fig. 3) is at least consistent with the predictions of this model (cf. refs. 19 and 20).

The coexistence of hydrophilic and hydrophobic pathways of diffusion in a single biological membrane was proposed by Sha'afi et al. [21] for erythrocytes in order to explain seemingly paradoxical data for the penetration of various small molecules. The duality of the penetration mechanisms is more easily seen with bacterial outer membrane, where pore diameters are presumably large, and where dissolution into the membrane interior would obviously be very difficult for substances such as oligosaccharides, which would have to break many hydrogen bonds to leave the aqueous environment. Another significant advantage of the outer membrane system is the availability of genetic mutants, and this advantage was exploited in this study.

It should not be assumed that any one property of the solute, be it molecular size or hydrophobicity, consigns the solute exclusively to one or the other pathway of penetration. For example, a number of substances in group II have molecular weights lower than 600, and thus should be able to diffuse through pores. Perhaps the ability of a solute to form hydrogen bonds influences its rate of passage through pores [21]. Penecillin derivatives, for example, should form far fewer hydrogen bonds than oligosaccharides with similar molecular weights, and thus might penetrate much more slowly through aqueous pores.

Interestingly, a converse situation is found with a few group I agents which seem to utilize hydrophilic pathway in spite of their high partition coefficients. Tetracycline and chlortetracycline, however, appear to enter cytoplasm as complexes with divalent cations [22], and might also penetrate the outer membrane as (presumably more hydrophilic) Mg^{2+} complexes. It is not clear why chloramphenicol prefers the aqueous pathway; possibly it might utilize a specific penetration mechanism, a few examples of which are known to exist in the outer membrane [23–25].

Group II agents contain two exceptional substances with low partition coefficients (Table I). Polymyxin, however, interacts specifically with lipopolysaccharide [26], and its partition coefficient in the octanol system may not reflect its true affinity toward the interior of the outer membrane. For vancomycin, the slow diffusion through the hydrophobic pathway is probably the only possible avenue of penetration because of its large size.

It should be emphasized that the outer membrane of wild type *Salmonella* hardly allows any penetration of hydrophobic molecules. The "hydrophobic pathway of penetration" therefore is an artefact which operates only in laboratory-produced deep rough or omp mutants. Undoubtedly the normal barrier must be of enormous benefit to the organism, making it resistant to a number of hydrophobic antibiotics and, especially in the case of enteric bacteria, to fatty acids and bile salts. However, since biological membranes usually are quite permeable to hydrophobic substances [19–21], one wonders why the wild type outer membrane constitutes an exception in this respect. The simplest explanation is that the saccharide side chains of lipopolysaccharide form a hydrophilic layer on the outer surface of the membrane, and that the layer cannot be penetrated by the hydrophobic molecules. This hypothesis, however, does not explain why the deletion of 80–90 % of the sugar residues on the cell surface, as in Rc mutants, hardly affects hydrophobic penetration while the removal of just one more residue results in such a drastic change (compare Rc with Rd_1 in Table II). A more satisfactory explanation is suggested by our finding that no exposed phospholipid head group could be detected on the outer surface of the outer membrane of wild type, or Rc cells, by the use of a covalent labeling reagent as well as phospholipase C [27]. This could be a result of the complete absence of phospholipid molecules in the outer half of the outer membrane, or it could be that all head groups are buried under proteins. In any case, the lack of exposed phospholipid molecules could prevent the dissolution of hydrophobic molecules into the interior of the membrane.

In deep rough mutants, in contrast, the absence of the crucial glucose residue (Glc_1 of Fig. 1) apparently blocks the incorporation of many proteins into the outer membrane [9], and produces a compensatory increase in the phospholipid content of the outer membrane [28]; the head groups of much of the outer membrane phospholipids then become exposed, i.e. accessible to the labeling reagents from the outside medium [27]. The presence of these exposed phospholipid bilayer regions should then allow the rapid penetration of hydrophobic molecules, just as model phospholipid bilayers do [29, 30].

The omp mutants with reduced levels of outer membrane proteins [9] allow a rapid penetration of hydrophobic molecules (Table II) in spite of the presence of the normal amounts of Rc-type lipopolysaccharide. This result is also consistent with the idea that reduction in outer membrane proteins and the accompanying changes in membrane organization, rather than the alteration of lipopolysaccharide structure per se, are the direct cause of increased hydrophobic permeability in the mutants.

It is known that *E. coli* cells lose a substantial fraction of lipopolysaccharide molecules upon brief treatment with EDTA [31], and at the same time become sensitive to actinomycin D [32]. It is conceivable that the extraction of lipopolysaccharide results in the reorganization of the membrane with the production of exposed phospholipid bilayer regions; such a possibility is currently under investigation.

The working hypotheses proposed above on the mechanisms of penetration and on the organization of the outer membrane may not turn out to be entirely correct. Nevertheless, the observation summarized in Table I tells us that the efficacy of any hydrophobic agent against *Salmonella* or *E. coli* is likely to be much higher if deep rough mutants are used. This knowledge should be of value in studies involving

the use of hydrophobic agents or effectors. Indeed, upon the author's suggestion, Ames et al. [33] have successfully used deep rough mutants in order to make *Salmonella* strains susceptible to the mutagenic action of polycyclic hydrocarbons.

An understanding of outer membrane permeability is also important in designing antibiotics and chemotherapeutic agents against Gram-negative bacteria. In fact, a survey of penicillin derivatives [34, 35], for example, indicates that the effectiveness of derivatives can usually be predicted on the basis of the observations described in this study. Thus any alteration of the molecule which makes the molecule more hydrophilic, or increases its hydrogen bonding ability, is likely to expedite the penetration through the pores and to make the derivative more effective toward Gram-negatives. The addition of bulky, hydrophobic substituents inhibits the penetration, since hydrophilic pores are essentially the only non-specific permeation pathway operating in the wild type cells of Gram-negative bacteria.

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