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Porin Channels in *Escherichia coli*: Studies with β-Lactams in Intact Cells

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Wild-type *Escherichia coli* K-12 produces two porins, OmpF (protein 1a) and OmpC (protein 1b). In mutants deficient in both of these "normal" porins, secondary mutants that produce a "new" porin, protein PhoE (protein E), are selected for. We determined the properties of the channels produced by each of these porins by measuring the rates of diffusion of various cephalosporins through the outer membrane in strains producing only one porin species. We found that all porin channels retarded the diffusion of more hydrophobic cephalosporins and that with monoanionic cephalosporins a 10-fold increase in the octanol-water partition coefficient of the solute produced a 5- to 6-fold decrease in the rate of penetration. Electrical charges of the solutes had different effects on different channels. Thus, with the normal porins (i.e., OmpF and OmpC proteins) additional negative charge drastically reduced the penetration rate through the channels, whereas additional positive charge significantly accelerated the penetration. In contrast, diffusion through the PhoE channel was unaffected by the presence of an additional negative charge. We hypothesize that the relative exclusion of hydrophobic and negatively charged solutes by normal porin channels is of ecological advantage to *E. coli*, which must exclude hydrophobic and anionic bile salts in its natural habitat. The properties of the PhoE porin are also consistent with the recent finding (M. Argast and W. Boos, J. Bacteriol. **143**:142–150, 1980; J. Tommassen and B. Lugtenberg, J. Bacteriol. **143**:151–157, 1980) that its biosynthesis is derepressed by phosphate starvation; the channel may thus act as an emergency pore primarily for the uptake of phosphate and phosphorylated compounds.

Gram-negative bacteria, including *Escherichia coli*, are covered by outer membranes, so that nutrients as well as antibiotics must first penetrate through these membranes before they reach their final destination or target. Fractionation and in vitro reconstitution of active principles, as well as the use of mutants deficient in the presumptive active component, have led us to conclude that most nutrients and most antibiotics penetrate the outer membrane of *E. coli* and related organisms through water-filled channels produced by a class of proteins called porins (4, 15, 19). In a previous study, we have shown that the rates of penetration of hydrophilic, uncharged solutes through the *E. coli* channel were very strongly dependent on the size of the solute, an observation that led to the estimation of the pore diameter at about 1.2 nm (21). In a further attempt to characterize the properties of the porin channel, we examined the effect of solute hydrophobicity and charge on penetration rates through the channel. For this purpose, we needed a group of closely related compounds differing in hydrophobicity and charge and also methods for measuring the diffusion rates of these compounds across the outer membrane. Both of these conditions were fulfilled by semisynthetic β-lactam compounds, as thousands of these compounds have been synthesized (17, 29) and measurement of their permeation rates recently became feasible (30, 39).

Another point emphasized in the present study is the comparison of the properties of channels produced by different porins. *E. coli* K-12 normally produces two species of porin, 1a and 1b, coded by *ompF* and *ompC* genes, respectively (2, 3, 7, 32, 38). (These proteins will be called OmpF and OmpC, respectively.) Furthermore, the relative expression of these two proteins is regulated by environmental conditions (3, 16), and this observation suggested that the properties of OmpF and OmpC channels might be different. In addition, some mutants deficient in normal porins generate "second-site

suppressor mutants" in which a normally repressed "new" porin becomes derepressed (10, 25); the channel produced by such a new porin, protein E (also called Ic and e, recently shown to be coded for by gene *phoE* [36], and therefore called PhoE in this paper), present in *nmpA* mutants, has been included in this comparison.

MATERIALS AND METHODS

Organisms. All strains used were derivatives of *E. coli* JF568 (K-12 *aroA357 ilv-277 metB65 his-53 purE41 cyc-1 xyl-14 lacY29 rpsL77 tsx63*) and produced only one species of porin. Thus, JF701 (JF568 *ompC264*) produced only the OmpF porin, JF703 (JF568 *ompF254*) produced only the OmpC porin, and JF694 (JF568 *ompC264 ompF254 nmpA1*) produced only the PhoE porin (8). In addition, an R-factor specifying the production of a TEM-type, periplasmic β-lactamase, R_{471a} (12), was transferred to these strains by conjugation from YC215 (*E. coli* K-12 *lac gal mtl xyl ara*/R_{471a}, a gift of M. Yoshikawa). R_{471a} was chosen because it caused the production of very high levels of β-lactamase activity, more than an order of magnitude higher than that obtained by the incorporation of such plasmids as R1 (12). The stock cultures were maintained at −70°C, and preculture was performed in the presence of 100 μg of ampicillin per ml, to prevent the loss of the plasmid. The extracts of the three strains contained about equal levels of β-lactamase; this indicates that we did not get preferential loss of plasmids from any of these strains.

Cephalosporins. Cephaloridine, cefazolin, cefamandole, cephalothin, cephaloglycin, and cephaloram, as well as the experimental compound 7-[2-(2-benzothienyl)acetamido]cephalosporanic acid (benzothienylcephalosporin), were gifts of Lee F. Ellis, Eli Lilly & Co., Indianapolis, Ind. Cephacetrile and cephapirin were donated by W. Zimmermann, CIBA-Geigy, Basel, Switzerland, and F. Leitner, Bristol Laboratories, Syracuse, N.Y., respectively. SCE-20 and cefsulodin were gifts from H. Nomura, Takeda Pharmaceutical Co., Osaka, Japan. The structures of these compounds are shown in Fig. 1.

Assay for hydrolysis of cephalosporins. Because the absorption of cephalosporins in the vicinity of 260 nm is altered drastically by the cleavage of the β-lactam ring (9, 18), continuous recording of optical density in this range provided a convenient and accurate method for following the hydrolysis of cephalosporins. However, when hydrolysis by intact cells was measured, the extensive scattering of light by these cells contributed very strongly to the background, making the assay nearly impossible. We circumvented this difficulty by using cuvettes of 1-mm light path. This allowed us to use 10-fold-higher concentrations of both cells and cephalosporins than would have been possible with the standard, 10-mm cuvette. Since the rates of hydrolysis by intact cells are largely limited by diffusion rates through the outer membrane, which in turn are more or less proportional to the external concentration of cephalosporins, under these conditions the rate of hydrolysis is accelerated by a factor of 10 × 10, or 100. Although we lose photometric sensitivity by a factor of 10, the overall effect of using the 1-mm cells is an approximately 10-fold gain in the sensitivity of the assay, which enabled us to follow accurately the hydrolysis of slowly degraded cephalosporins by intact cells.

The practical arrangement for the experiments was as follows. Cells of bacterial strains carrying the R plasmid were grown and washed as described previously (20). A portion of the cell suspension (5 mg [dry weight] ml^{-1}) was sonicated as described before (20), and the sonic extract was used for the determination of V_{max} (and in preliminary experiments also the K_m) of the periplasmic β-lactamase. Intact cells (0.15 mg [dry weight]) or extracts were added to an assay medium containing 10 mM sodium phosphate buffer (pH 6.0)–5 mM $MgCl_2$–1 mM cephalosporin (final volume, 0.5 ml). The suspension was then mixed and rapidly transferred to a cuvette with a 1-mm light path, and the optical density was recorded at 260 nm with a Perkin-Elmer-Hitachi model 124 spectrophotometer. Assays were performed at 25°C, and the diffusion rates of β-lactams across the outer membrane were calculated essentially according to the method of Zimmermann and Rosselet (39). K_m values of R_{471a} β-lactamase were as follows: cephacetrile (1,350 μM), cefazolin (410 μM), cefamandole (780 μM), cephalothin (310 μM), cephaloram (280 μM), benzothienylcephalosporin (1,540 μM), cephaloridine (930 μM), cephaloglycin (680 μM), SCE-20· (780 μM), and cefsulodin (540 μM).

An example of calculation of the permeability coefficient (P) is given below. When the sonic extract from 0.15 mg (dry weight) of cells of JF701 (R_{471a}) was incubated in a 0.5-ml assay mixture containing 1 mM cephalothin, the optical density at 260 nm decreased with an initial rate of 0.109 min^{-1}. In a separate experiment, when the hydrolysis of various cephalosporin derivatives that were nominally 1 mM was followed to completion, the total change in optical density at 260 nm was found to be between 0.6 and 0.8. In view of the uncertainty regarding the purity of some of these compounds, we assumed that the complete hydrolysis of a 1 mM solution of any cephalosporin would decrease the optical density at 260 nm of the solution by 0.8 in a cell of 1-mm light path (9). Thus, the rate of hydrolysis by an extract from 1 mg of cells (V_{ext}) is $(0.109 \div 0.8 \div 0.15) \times 0.5 \times 10^3 = 454$ nmol min^{-1}. Since the K_m of the R_{417a} β-lactamase for cephalothin is 310 μM, from the Michaelis-Menten relationship, $V_{max} = V_{ext}\,(1{,}000 + K_m)/1{,}000 = 595$ nmol min^{-1}. When intact cells (0.15 mg [dry weight]) were incubated similarly with 1 mM cephalothin, the rate of decrease of optical density at 260 nm was 0.027 min^{-1}, corresponding to the rate of hydrolysis of 113 nmol min^{-1} mg^{-1}. When we substitute this rate (V_{cells}), the V_{max} value obtained above, and the K_m into the Michaelis-Menten equation, we obtain a substrate concentration in the periplasmic space (C_p) of 73 μM (or 73 nmol cm^{-3}). According to Fick's first law, $V_{cells} = P \cdot A \cdot (C_o - C_p)$, where A and C_o represent the area of cell surface per unit weight and the concentration of the β-lactam in the external medium, respectively. From the values of V_{cells}, A (132 cm^2 mg^{-1}; reference 33), C_o (1,000 nmol cm^{-3}), and C_p, P can be estimated as (113 nmol min^{-1} mg^{-1} ÷ 132 cm^2 mg^{-1}) ÷ (1,000 − 73 nmol cm^3) = 9.2×10^{-4} cm min^{-1} or 1.5×10^{-5} cm s^{-1}.

Determination of the octanol-water partition coefficient. Partition coefficients of the unionized forms (P_u)

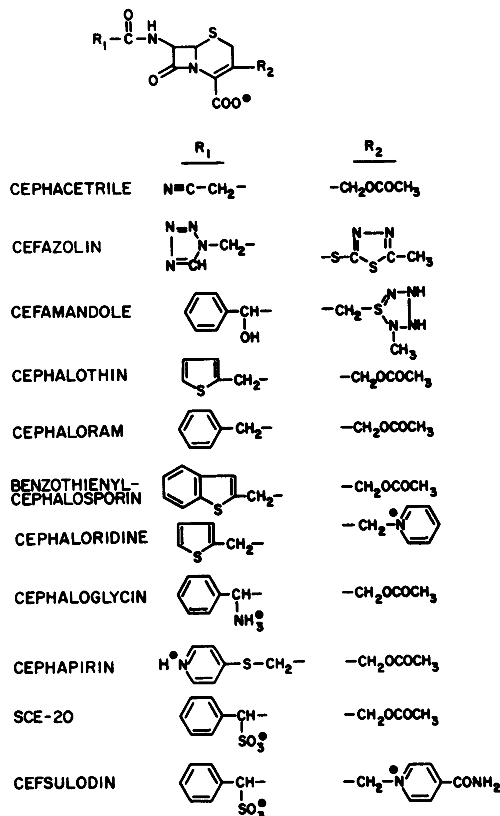

FIG. 1. Structures of cephalosporins used in this study.

of monobasic cephalosporins were determined as described by Tsuji et al. (37). Briefly, 1 mM solutions of cephalosporin were made in 0.1 M glycine-HCl buffer, pH 2.0 or 3.0, or in 0.1 M sodium citrate buffer, pH 4.0, and after vigorous shaking with an equal volume of 1-octanol at 25°C, the concentrations of the drug in the organic and aqueous phases were determined by spectrophotometry. The values of the apparent partition coefficients (P_{app}) obtained in this manner were multiplied by $([H^+] + K_a)/K_a$, where $[H^+]$ and K_a denote the hydrogen ion concentration in the aqueous phase and the dissociation constant of the cephalosporin, respectively, and the product was plotted against $[H^+]/K_a$. The data points form a straight line, and the slope of the line corresponds to P_u (37). K_a was obtained from the literature (37) for cephalothin and cefazolin. For other compounds, the exact pK_a values were unknown. However, the nature of side chains produced very little difference in pK_a values of cephalosporins (9), and we found that an error of 0.5 pH unit in the assumed pK_a value produced only an error of about 0.05 in the log P_u value obtained. Thus, we assumed the pK_a of 2.4 for all other monobasic cephalosporins. The values of log P_u obtained for cephacetrile, cefazolin, cefamandole, cephalothin, cephaloram, and benzothienylcephalosporin were −0.45, −0.24, 0.50, 1.09, 1.31, and 1.68, respectively. The P_u for cefazolin was calculated by using P_{app} values obtained only at pH 4.0 and 3.0, because at pH 2.0 the effect of the protonation of the side chain (pK_a = 1.7; see reference 37) became apparent.

Calculations of P_u from chemical structure. The experimental determination of P_u was very difficult or sometimes impossible with cephalosporins containing more than one charged groups. Furthermore, in some of the comparisons, P_u values for hypothetical compounds were required (see Results). The P_u values were therefore calculated by a procedure based on the additivity of hydrophobic interactions (11, 26). As an example, the P_u value for cephaloglycin was calculated as follows. Since cephaloglycin contains a phenyl group and a $CHNH_2$ group instead of the thienyl group-plus-CH_2 group in the cephalothin, from the "fragmental constants" of phenyl group (1.90), CH (0.24), NH_2 (−1.38), thienyl group (1.59), and CH_2 (0.53) (26) and the measured P_u of cephalothin (1.09; see above), we can calculate the P_u of cephaloglycin to be $1.09 - 1.59 - 0.53 + 1.90 + 0.24 - 1.38 = -0.27$. The P_u values of other compounds were calculated similarly by using the measured P_u of cephalothin as the starting point. Calculated P_u values for cephaloram and benzothienylcephalosporin were 1.40, and 1.86, respectively, and were reasonably close to the experimentally determined values of 1.31 and 1.68; this agreement suggested the general reliability of these calculations.

Determination of critical micellar concentrations. The shift in the absorption maximum of rhodamine 6G was used as evidence for micelle formation (6). The cephalosporins were dissolved in 10 mM sodium phosphate buffer, pH 6.0, and the absorption spectra were recorded at room temperature with a Perkin-Elmer-Hitachi model 124 spectrophotometer.

RESULTS

Experimental approach. We have used the Zimmermann-Rosselet method (39) of determining the permeability of the outer membrane from the rates of hydrolysis of β-lactams by intact cells. Since the intrinsic β-lactamase activity of *E. coli* K-12 strains is very weak, we had to introduce an R-factor containing a β-lactamase gene. R_{471a} was chosen as an R-factor that produces very high levels of TEM-type β-lactamase (12).

Because the host cell has been modified by the introduction of the R-factor, it was important to know that the pores in the outer membrane were not altered by this process. We believe that this is a correct assumption because of the following. (i) The protein composition of the outer membrane was not altered noticeably by the introduction of the R-factor (Fig. 2). (ii) The introduction of the R-factor into an *E. coli* mutant producing greatly diminished amounts of porin (4) did not change the diffusion rates of sugars across the outer membrane (Nikaido and Rosenberg, unpublished data), a result indicating that the R-factor does not produce additional nonspecific channels. (iii) The strains containing R_{471a} maintained the same sensitivity level to various antibiotics, such as tetracycline, chlor-

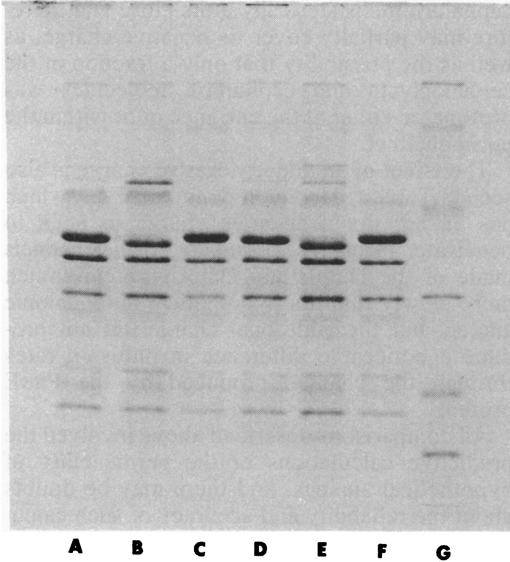

FIG. 2. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of outer membrane proteins. Outer membranes were prepared from various strains grown in L broth, as described previously (33), and samples containing 15 μg of protein each were analyzed by electrophoresis as described by Lugtenberg et al. (14). Lane A, JF-701 (containing OmpF only); B, JF703 (containing OmpC only); C, JF694 (containing PhoE only); D, JF701 (R_{471a}); E, JF703 (R_{471a}); and F, JF694 (R_{471a}). Molecular weight standards (phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400) were applied to lane G. Scanning of this Coomassie blue-stained gel indicated that the amount of OmpC protein in JF703 (R_{471a}) was 70% of the amount of OmpF protein present in JF701 (R_{471a}). Although a band with an apparent molecular weight of 50,000 (probably the phage lambda receptor) is more intense in lane B than in lane E, this difference was not reproducible in other experiments.

amphenicol, and streptomycin (data not shown). (iv) The permeability characteristics determined in cells containing R_{471a} in this paper are very similar to those determined by the use of porins purified from cells not containing the R-factor, described in the accompanying paper (22).

The major problems in the determination of cephalosporin hydrolysis by intact cells were that the rates were frequently very slow and even small amounts of β-lactamase released into the medium introduced large errors in the calculation of permeability coefficients, especially for slowly penetrating drugs. As described above, the first problem was solved by the use of spectrophotometric cuvettes of 1-mm light path, and the second was solved by growing cells in a medium containing 5 mM $MgSO_4$ and carrying out the assay also in the presence of 5 mM Mg^{2+}

Effect of hydrophobicity on rates of penetration. When a series of monoanionic cephalosporins were used, we found large differences in their rates of penetration through the outer membrane. Furthermore, the rates appeared to be strictly dependent on the hydrophobicity of the molecule, a 10-fold increase in P_u producing about a 5- to 6-fold decrease in the permeability coefficient (Fig. 3). The dependence on hydrophobicity was similar among OmpF, OmpC, and PhoE porins, but cells containing only the OmpC porin showed consistently about a 5-fold-lower permeability than those containing the OmpF porin. This cannot be entirely due to the difference in the number of porin molecules per cell, because differences in the porin contents of JF701 and JF703 were not large (Fig. 2).

Effect of positive charge on rates of penetration. Zimmermann and Rosselet (39) noted that cephaloridine diffused across the outer membrane much faster than expected from its apparent partition coefficient. Since cephaloridine is zwitterionic, unlike the other cephalosporins they tested, this result suggests that the additional positive charge may accelerate the diffusion process. However, the apparent partition coefficient is influenced greatly by charges, and thus it does not accurately reflect the hydrophobicity of the molecule. We tried to separate the effect of

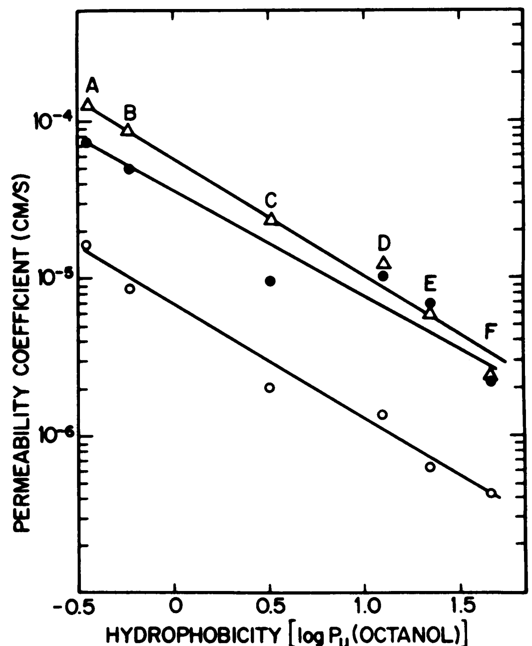

FIG. 3. Permeability coefficients of *E. coli* outer membrane to various monoanionic cephalosporins. Symbols: △, JF694 (R_{471a}) containing PhoE porin; ●, JF701 (R_{471a}) containing OmpF porin; ○, JF703 (R_{471a}) containing OmpC porin. For other details, see text.

charges from that of hydrophobicity by using P_u, i.e., the partition coefficient of the uncharged form, for correlation between the penetration rates and properties of the solute. In the case of cephaloridine, the experimental determination of P_u is impossible because it is zwitterionic and because the positive charge is carried by the quaternary nitrogen atom. We therefore had to calculate the approximate value of its P_u from its structure, as described in Materials and Methods. With its P_u value known, reference to Fig. 3 enabled us to predict the permeability coefficients for the monoanionic analog of cephaloridine. When the predicted permeability coefficients for the hypothetical monoanionic analog were compared with the observed coefficients for the zwitterionic cephaloridine, it was clear that the positive charge indeed resulted in a >100-fold acceleration of penetration through OmpF and OmpC channels, but the diffusion through the PhoE channel was affected much less (Table 1).

Similar comparison between the measured permeability coefficients of the zwitterionic compounds cephaloglycin and cephapirin and those predicted for their hypothetical monoanionic analogs again showed the accelerating effect of the positive charge in OmpF and OmpC channels (Table 1). The magnitude of the effect, however, was much less than with cephaloridine. This may be because the positive charge of cephaloridine is sterically quite close and therefore may partially cover its negative charge, as well as the possibility that only a fraction of the cephaloglycin and cephapirin molecules was protonated either at the entrance of or within the porin channel.

The effect of an added negative charge is also seen in Table 1. Here compound SCE-20, which has an additional negative charge, is seen to penetrate through "normal" porin channels made of the OmpF and OmpC proteins much more slowly than its hypothetical monoanionic analog, but the additional charge did not produce a noticeable difference in diffusion rates through the channel produced by the PhoE protein.

All comparisons described above involved the predictive calculations of the permeability of hypothetical analogs, and there may be doubts about the reliability and accuracy of such calculations. In one situation, however, we could compare the measured permeability coefficients of two real compounds. This comparison (Table 2) involves cefsulodin and cephaloridine, and it is seen that the former compound penetrates 20 to 30 times more slowly than the latter through the normal porin channels. A major part of this difference is almost certainly due to the presence of the additional negative charge on cefsulodin (see Fig. 1). Although there are two other differences between the two agents, the benzene and

TABLE 1. Effect of additional positive or negative charges on permeability of β-lactams through porin channels

β-Lactam	Electrical charge	Permeability coefficients (10^{-5} cm s^{-1}) in cells producing only porin:		
		OmpF	OmpC	PhoE
Cephaloridine (A)	+ −	52.6	4.5	2.5
Monobasic analog (B)[a]	−	0.08	0.01	0.08
(Ratio A/B)		(658)	(450)	(31)
Cephaloglycin (A)	+ −[b]	9.8	1.0	1.0
Monobasic analog (B)	−	4.7	0.8	7.5
(Ratio A/B)		(2.1)	(1.3)	(0.1)
Cephapirin (A)	+ −[c]	19.4	1.2	1.3
Monobasic analog (B)	−	3.2	0.6	5.0
(Ratio A/B)		(6.1)	(2.0)	(0.3)
SCE-20 (A)	− −	0.29	0.04	1.6
Monobasic analog (B)	−	1.3	0.12	1.7
(Ratio A/B)		(0.2)	(0.3)	(0.9)

[a] The expected permeability coefficients for the hypothetical monobasic analogs were calculated as follows. First, the log P_u value for the β-lactam with an identical structure but with only one possible negative charge at the 4-carboxyl position was calculated from the structure as described in the text. The permeability coefficients were then predicted from the lines of Fig. 3, on the basis of this value of log P_u.

[b] From the reported pK_a (6.8) of the side-chain amino group (36), about 88% of this group should be protonated under the conditions of assay, i.e., at pH 6.0.

[c] From the pK_a (5.94) of an analog, 4-methylthiopyridine (13), of the side-chain portion of the molecule, a substantial fraction of cephapirin molecules is expected to be in the zwitterionic form.

TABLE 2. Permeability coefficients of cefsulodin and cephaloridine through porin channels

β-Lactam	Electrical charge	Permeability coefficients (10^{-5} cm s^{-1}) in cells producing only porin:		
		OmpF	OmpC	PhoE
Cephaloridine (A)	+ −	52.6	4.5	2.5
Cefsulodin (B)	+ − −	1.8	0.2	3.2
(Ratio A/B)		(29.2)	(22.5)	(0.8)

the thiophene rings are of about the same size and hydrophobicity (11, 26) and the $-CONH_2$ group present on cefsulodin should significantly accelerate, rather than retard, the diffusion by decreasing log P_u by as much as 1.1 (26). Indeed, we observed some acceleration of the penetration of cefsulodin through the PhoE porin channel (Table 2). These results then confirm the conclusions obtained by the use of predicted permeability values of hypothetical compounds.

DISCUSSION

In this study we have examined the permeability coefficients of various cephalosporins to increase our knowledge of the properties of the porin channel in the outer membrane of *E. coli*. For this approach, we had to be certain that the cephalosporins were passing through the porin channel, i.e., not through the lipid bilayer region of the membrane or channels produced by other proteins. We feel that this condition was satisfied in our system on the basis of the following lines of evidence. (i) With the usual biological membranes, hydrophobic molecules, especially nonelectrolytes, can easily traverse the membrane through the bilayer continuum of the membrane. However, the lipopolysaccharide-phospholipid region of the outer membrane of *E. coli* and *Salmonella typhimurium* is unusually impermeable and indeed did not allow the penetration of even an extremely hydrophobic β-lactam, presumably due to the asymmetric arrangement of the lipopolysaccharide in the membrane (19). Furthermore, the ubiquitous negative charge present in all cephalosporin molecules (Fig. 1) should act as a deterrent against diffusion through this pathway. (ii) Mutants with trace levels of porin showed only 5 to 10% of the permeability of the wild-type cells to various β-lactams, including 6-aminopenicillanic acid (4), cephaloridine (23), cephacetrile, cefazolin, and cephalothin (Nikaido, unpublished data). (iii) Liposomes containing only the purified porin proteins showed permeability properties (22) similar to those observed with intact cells in this study.

Permeability coefficients of monoanionic cephalosporins, or thus their rates of diffusion through porin channels, showed a monotonous dependence on the degree of hydrophobicity of the solute (Fig. 3). It is not clear why hydrophobic molecules penetrate more slowly through the porin channels, but the following explanation seems plausible. Since the porin channels are very narrow (equivalent to a hollow cylinder of about 1.2 nm in diameter [21]), it is expected that water molecules in the channel are rather strongly bound to groups on the walls of the channel through hydrogen bonds. When hydrophilic solutes, which possess many hydrogen bond-forming groups on their surface, come into the channel, the hydrogen bonds between water molecules and groups on the wall are broken, but they are now replaced by new hydrogen bonds between the solutes and the wall, so that the net energy requirement will be minimal. In contrast, hydrophobic solutes will break many hydrogen bonds in the channel without replacing them with new hydrogen bonds; thus, the penetration of hydrophobic solutes would be an energetically unfavorable process. An alternative explanation is that the more hydrophobic cephalosporins tend to form micelles, which are too large for penetration through the porin channel. However, our assay (see Materials and Methods) has shown that none of the cephalosporins used was in micellar form at the concentration used (1 mM) at the pH of assay (6.0) (data not shown).

The cephalosporins used in the experiment of Fig. 3 had molecular weights ranging from 338 to 451. Since the size of the solutes had a very strong influence on their rates of penetration through the porin channels (21), it was rather unexpected to find that the penetration rates were determined mostly by hydrophobicity and were affected much less by the molecular weights of the agents (Fig. 3). However, building of space-filling models showed that the sizes of most cephalosporins varied much less than expected from the difference in molecular weights. The observation that cefamandole and cefazolin tended to have lower diffusion rates than those predicted from their hydrophobicity could be a reflection of their somewhat larger sizes (Fig. 1 and 3).

The channels made of OmpF, OmpC, and PhoE porins behaved similarly toward the hydrophobicity of the solute (Fig. 3). However, the absolute values of permeability coefficients in OmpF-containing cells was always several times higher than those in OmpC-containing cells. This difference cannot be ascribed to the difference in copy numbers of these porins, as sodium dodecyl sulfate-polyacrylamide gels of the outer membranes of OmpF- and OmpC-containing strains showed that the levels of these porins were not so different in these cells (Fig. 2).

Although it is possible that fractions of channels that are in "open" conformation (31) differ in these proteins, a simpler explanation of this difference in the magnitude of permeability coefficients is the slight difference in the equivalent diameter of OmpF and OmpC channels, as discussed in the accompanying article (22).

The monotonic relationship between the hydrophobicity and the permeability coefficient (Fig. 3) allowed us to predict the permeability of any hypothetical monoanionic cephalosporins, and this method was used to study the effect of electrical charges on penetration rates. The results showed that with channels produced by the normal *E. coli* porins OmpF and OmpC, the additional positive charge accelerated the diffusion process, whereas the presence of the additional negative charge significantly retarded the penetration rates. These channels thus appear to prefer cations. An alternative explanation is that the Donnan potential (interior negative) across the outer membrane (34) is pulling the cations in. However, the channel itself seems to have some discriminatory power, as indicated by the following observations. (i) In reconstitution studies with black lipid film, it was also found that the *E. coli* porin channel preferred alkali metal cations to the chloride anions (5). (ii) Retardation of negatively charged molecules was also confirmed in liposome systems (22). (iii) In spite of the Donnan potential, the PhoE porin channel seems to favor negatively charged solutes (see below).

The channels made of PhoE porin, whose production is normally repressed in wild-type *E. coli*, were very different from those of OmpF and OmpC channels. Here the presence of an additional positive charge produced either significant retardation or only a small acceleration (Table 2). The addition of the second negative charge did not result in a significant retardation (Tables 1 and 2). Thus, the PhoE channel appears to be a channel that tolerates, and even prefers, multivalent anions. This conclusion is in agreement with the recent observation that the expression of PhoE porin occurs under phosphate starvation conditions (1, 35); probably the PhoE porin is an anion-preferring channel permitting the rapid diffusion of a wide range of negatively charged solutes.

After the first version of this paper was completed, a paper by Overbeeke and Lugtenberg (24) was published. Their results are generally consistent with ours as regards the perference of the PhoE channel for negatively charged solutes. However, these workers also noted that 2.5 mM Na_2HPO_4 added to intact cells at pH 7.0 reduced the diffusion rate of cefsulodin through the PhoE channel by 40% (24). Because we used 10 mM sodium phosphate buffer in our assay, there was a possibility that the diffusion through the PhoE channel could have been inhibited by the phosphate ions in the buffer. The assays were therefore repeated with PhoE-, OmpC-, and OmpF-containing cells in 10 mM sodium phosphate buffer, pH 6.0, as well as in 10 mM bis-(2-hydroxyethyl)amino-tris(hydroxymethyl) methane–hydrochloride buffer, pH 6.0. With the OmpC- and OmpF-containing cells, we did not observe any inhibition by phosphate buffer with several substrates. With the PhoE-containing cells, the diffusion rates were decreased in phosphate buffer, but only by 8% for cephaloridine and 15% for cefsulodin. (The values are averages of four independent experiments.) Whatever the reasons for the discrepancy, these results suggest that our conclusions on the properties of the pores remain essentially valid.

Since only *E. coli* and *S. typhimurium* (and probably their close relatives), among the gram-negative species so far surveyed (19), have been found to contain the narrow porin channels that exclude hydrophobic and anionic molecules, we should briefly consider the possible physiological significance of the channels with these properties. We feel that one of the most serious challenges *E. coli* faces in the upper intestinal tract is the presence of high concentrations of bile salts, which are powerful detergents and for this reason inhibit the growth of most nonenteric bacteria on selective media such as McConkey and deoxycholate agars. The *E. coli* porin channels may have evolved in such a way as to exclude bile salts, which are hydrophobic, rather bulky, and negatively charged.

Although the main aim of this study was to understand the properties of the porin channel, some comments would be appropriate as regards the permeation of β-lactams in gram-negative bacteria, since these agents cross the outer membrane through porin channels and since great efforts have been made to produce compounds with faster rates of penetration. Our observations can be summarized as follows. (i) Some of the β-lactams tested, for example, cephaloridine and cephacetrile, penetrate through the channels quite rapidly, and their permeability coefficients (1×10^{-4} to 5×10^{-4} cm s^{-1} through the OmpF porin channel) can be favorably compared with those of uncharged, very hydrophilic molecules of about the same size, e.g., about 2×10^{-4} cm s^{-1} for lactose (21). It would be difficult to produce further dramatic improvement on penetration rates, unless a net positive charge is introduced. (ii) The negative effect of hydrophobicity confirms earlier results from several other laboratories. For example, it was shown repeatedly that the more hydrophobic β-lactams tended to be less effective against *E. coli* (17, 29), although the correlations were

less than perfect as modification of the drug also alters its affinity to the target sites. Zimmermann and Rosselet (39) showed, for the first time, the correlation between hydrophobicity of the β-lactams and their penetration rates, although the effect of electrical charges was not separated from the effect of hydrophobicity. (iii) Since our values of permeability are expressed in permeability coefficients rather than in arbitrary units used by other workers, we can calculate $t_{1/2}$, the time necessary for the intracellular concentration of β-lactam (C_i) to reach 50% of the extracellular concentration (C_o). Fick's first law states, $d(C_i \cdot V)/dt = P \cdot A \cdot (C_o - C_i)$, where V is the volume of cells. Rearrangement, integration, and substituting $C_i = 0$ at $t = 0$ lead to: $t = V \cdot \ln(C_o/(C_o - C_i))/(P \cdot A)$. At $t = t_{1/2}$, $C_i = C_o/2$, and therefore, $t_{1/2} = \ln 2 \cdot V/(P \cdot A)$. Since we know that $A = 132$ cm^2 for cells of 1 mg of dry weight (33), i.e., about 4 mg of wet weight or about 4×10^{-3} cm^3 cell volume, $t_{1/2}$ can be obtained for any value of P. This calculation shows that for β-lactams of the most permeable group ($P \simeq 10^{-4}$ cm s^{-1}), $t_{1/2}$ is about 0.1 s, and even for very poorly penetrating β-lactams ($P \simeq 10^{-6}$ cm s^{-1}; see Fig. 3) $t_{1/2}$ is only about 20 s. Thus, the β-lactams penetrate surprisingly rapidly through *E. coli* outer membranes. The impressions of slow penetration created by the difference in minimal inhibitory concentrations between normal and "superpermeable" strains (27), for example, must therefore be due to the rapid hydrolysis, or binding, of β-lactam molecules that have crossed the outer membrane barrier. We should thus keep in mind that some of the "permeability assays" of the outer membrane may be measuring the balance between penetration and hydrolysis rather than the actual penetration rates. In fact, one of these assays indicates that both cephaloridine and cephalothin diffuse "unhindered" through the *E. coli* outer membrane and that cefamandole is much less permeable than caphalothin (28). These results are incompatible with our conclusions and, in our analysis, illustrate the basic flaw in the design of these assays. The magnitudes of the $t_{1/2}$ values also suggest that β-lactams that are very resistant to enzymatic hydrolysis can be quite effective toward gram-negative bacteria, even if their penetration through the outer membrane is rather slow. Some of the more recently developed β-lactams indeed appear to fall into this category (Nikaido, manuscript in preparation).

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