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Porin Channels in *Escherichia coli*: Studies with Liposomes Reconstituted from Purified Proteins

HIROSHI NIKAIDO* AND EMIKO Y. ROSENBERG

Department of Microbiology and Immunology, University of California, Berkeley, California 94720

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Rates of diffusion of uncharged and charged solute molecules through porin channels were determined by using liposomes reconstituted from egg phosphatidylcholine and purified *Escherichia coli* porins OmpF (protein 1a), OmpC (protein 1b), and PhoE (protein E). All three porin proteins appeared to produce channels of similar size, although the OmpF channel appeared to be 7 to 9% larger than the OmpC and PhoE channels in an equivalent radius. Hydrophobicity of the solute retarded the penetration through all three channels in a similar manner. The presence of one negative charge on the solute resulted in about a threefold reduction in penetration rates through OmpF and OmpC channels, whereas it produced two- to tenfold acceleration of diffusion through the PhoE channel. The addition of the second negatively charged group to the solutes decreased the diffusion rates through OmpF and OmpC channels further, whereas diffusion through the PhoE channel was not affected much. These results suggest that PhoE specializes in the uptake of negatively charged solutes. At the present level of resolution, no sign of true solute specificity was found in OmpF and OmpC channels; peptides, for example, diffused through both of these channels at rates expected from their molecular size, hydrophobicity, and charge. However, the OmpF porin channel allowed influx of more solute molecules per unit time than did the equivalent weight of the OmpC porin when the flux was driven by a concentration gradient of the same size. This apparent difference in "efficiency" became more pronounced with larger solutes, and it is likely to be the consequence of the difference in the sizes of OmpF and OmpC channels.

The outer membrane of a single gram-negative bacterium, such as *Escherichia coli*, contains up to 10^5 water-filled channels produced by proteins of a special class, called porins (21). So far two methods have been utilized in our laboratory for characterizing the properties of these channels. In the first method, porins are purified and then reconstituted into liposome vesicles made of phospholipids and lipopolysaccharides. The efflux of radiolabeled solutes through the porin channels is then determined by filtration through a gel filtration column (18). In the second method, the diffusion of solutes through the outer membrane is measured in intact cells by coupling the diffusion process with the process of solute removal, either by a periplasmic degradative enzyme (42) or by an active transport machinery located in the cytoplasmic membrane (22). Although the first method produced most of the early evidence that porins in fact produce channels, the time resolution of the method is poor and it has been impossible to measure penetration rates by this approach. The second method has been successful in producing the rate data, but the range of solutes that can be used is very limited. Furthermore, it is difficult to rule out the possibility that at least a fraction of the solute molecules is diffusing through nonporin pathways.

To circumvent these difficulties, a "swelling assay" that uses reconstituted liposomes was devised (14, 15, 22). In this assay, pure porin preparations are used, and thus we are certain that diffusion occurs only through the porin channel. Yet we can obtain the rates of solute penetration with reasonable precision from the rates of swelling of liposomes, induced by the influx of water accompanying the influx of uncharged solutes. In this study, we modified the assay procedure so that it can be used for measuring the diffusion of electrolytes and determined the effects of various properties of the solute molecule, including size, electrical charge, and hydrophobicity, on its penetration through channels produced by the three porins of *E. coli* K-12, coded by genes *ompF*, *ompC*, and *phoE* (21, 25, 38).

MATERIALS AND METHODS

Chemicals. Egg yolk phosphatidylcholine (type IX-E) was obtained from Sigma Chemical Co. and was freed of neutral lipid contaminants as follows (13).

Crude lipid (2.5 g) was crushed into a fine powder with mortar and pestle, and then 50 ml of acetone containing 2 mM mercaptoethanol was added. The extraction was continued for 3 h at room temperature with occasional crushing and kneading of the phospholipid with a pestle. The acetone extract was discarded, and the phospholipid was partitioned in the Folch system (8) with the addition of 1 mM EDTA instead of water and washed once with the theoretical upper-phase mixture (8). The lipid was stored, after drying with anhydrous Na_2SO_4, at −70°C.

Porins were purified as trimers from *E. coli* K-12 strains (JF701, JF703, and JF694), each of which produces only one species of porin (4, 23). The purification followed the procedure of Tokunaga et al. (37), except that a column of Sephacryl S-200 (Pharmacia Fine Chemicals, Inc.) was used for size fractionation. The porin preparations were more than 98% pure as judged by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (16) followed by scanning of stained gels. The final preparation was dialyzed for at least 2 weeks against 3 mM NaN_3, a treatment shown to produce porins containing <0.1% (by weight) sodium dodecyl sulfate (18). Phage lambda receptor protein (LamB protein) was purified in the same manner, except that a porin-deficient strain, T-19 (14), was used as the starting material.

The sources of other chemicals were as follows: NAD (free acid, grade III), Sigma; 2,3-diacetamido-2,3-dideoxyglucose, U.S. Biochemical Corp.; stachyose, gluconate, glucuronate, mucate, saccharate, lactobionate, 5'-AMP, and dicetylphosphate, Sigma. The sources of β-lactams are given in reference 20. Peptides were obtained from Serva, except *N*-formyl-L-phenyallanine (Sigma) and L-seryl-L-alanine (Vega). *N*-Acetyl-glycyl-glycyl-glycyl-glycine was prepared by the acetylation of tetraglycine, essentially according to reference 31. ^{3}H-labeled raffinose and stachyose were prepared in our laboratory (7). [^{3}H]sucrose and [^{14}C]-dextran were obtained from Amersham Corp. and New England Nuclear Corp., respectively.

For swelling experiments, all acids (including β-lactams) were converted into sodium salts. When the substance was provided as lithium, potassium, or calcium salt, these cations were first removed by passage through a small column (1 ml for 100 μmol of the salt) of Dowex 50 (H^+ form), and the solution was then neutralized with Na_2CO_3-free NaOH.

Liposome swelling assay. Since dextran-containing liposomes (14, 15) showed unexpected osmotic behavior towards electrolytes (see Results), the liposomes in this study were made in the presence of stachyose and NAD. However, the turbidity of the liposome suspension is dependent on the magnitude of the refractive index of the solution trapped inside the liposome vesicles, and the liposomes made in the low-refractive index solution containing stachyose had much lower turbidity than dextran-containing liposomes; therefore, we had to use larger amounts of phospholipids for swelling experiments. Usually, 6.2 μmol of acetone-extracted egg phosphatidylcholine (see above) and 0.2 μmol of dicetylphosphate (Sigma) were dried at the bottom of a tube, and the film was suspended in 0.2 ml of aqueous solutions of purified porin. Preliminary results indicated that the porins functioned fully in the environment of these "foreign" lipids; dicetylphosphate was added because phosphatidylcholine lacked the acidic phospholipids that were present in *E. coli* phospholipids. Addition of small amounts of lipopolysaccharide did not alter results, but our porin preparations could have contained traces of tightly associated lipopolysaccharides. The suspension was sonicated in a bath-type sonicator (Bransonic 12; Branson Cleaning Equipment Co.) until it became translucent (usually less than 15 s) and was then dried by connecting the tube to a vacuum pump equipped with a tube of drying agent ($CaSO_4$) and warming the tube in a water bath at 45°C. This drying step required about 1.5 min, and then the tubes were left overnight in the dark in an evacuated desiccator over $CaSO_4$. Finally, the film was suspended in 0.4 ml of a solution typically containing 12 mM stachyose, 4 mM sodium NAD, and 1 mM imidazole-NAD buffer, pH 6.0. The suspension was done by leaving the tubes without agitation at room temperature for 2 h and then hand shaking. The liposome suspension was then passed through an 8-μm membrane filter (Millipore Corp.) to remove large aggregates, 10 to 20 μl of the filtered suspension was diluted into 0.6 ml of test solutions, and the optical density was recorded at 400 nm with a Perkin-Elmer-Hitachi model 124 spectrophotometer at 25°C. The diluent solutions contained the following: (i) for testing permeability of nonelectrolytes, 1 mM imidazole-NAD (pH 6.0), 1 mM sodium NAD, and about 18 mM nonelectrolyte; (ii) for testing permeability of monovalent anions, 1 mM imidazole-NAD (pH 6.0), 1 mM sodium NAD, and approximately 9 mM sodium salt of the anion to be tested; (iii) for testing permeability of divalent anions, 1 mM imidazole-NAD (ph 6.0), 1 mM sodium NAD, and approximately 6 mM disodium salt of the anion to be tested. In all cases, the concentrations of the test solute were adjusted so that the diluent was apparently isotonic with control liposomes that did not contain porin. In some cases the osmotic activity of solutions was ascertained with a Wesco vapor pressure osmometer, made available through the courtesy of J. Forte. Furthermore, when solutions with widely different refractive indices were compared, Ficoll was added in appropriate amounts to make the refractive indices of the diluent solutions equal.

In some experiments liposomes were made in stachyose solutions containing imidazole buffer, to test the penetration of nonelectrolytes and zwitterionic solutes, and in sodium NAD solutions containing imidazole-NAD for measuring the permeation of anions. For example, for the measurement of cephalosporin anion diffusion, liposomes were made in 10 mM sodium NAD–0.5 mM imidazole-NAD buffer, pH 6.0, and were diluted into 10 mM solutions of sodium salts of β-lactams containing 0.5 mM imidazole-NAD, pH 6.0. Furthermore, with some hydrophobic β-lactams, results suggested that the protonated form of these solutes penetrated through the lipid bilayer region of the liposomes, followed by the penetration of Na^+ counterions through the porin channel (see Results). Thus, a separate control for the rate of diffusion of protonated β-lactams through the lipid bilayer was needed; for this purpose we used liposomes containing 1.5 μg of gramicidin A (catalog no. G5002, Sigma) per 6.2 μmol of phospholipids instead of porin. Gramicidin A forms an alkali-metal ion-specific channel (12), which thus allows the diffusion of counterions without permitting a facilitated entry of β-lactam molecules.

Other methods. The liposome filtration assay was carried out as described by Nakae (18). Other methods are given in previous publications from this laboratory (18, 19, 22–24).

RESULTS

Modification of the liposome swelling assay. The diffusion rates of solutes through porin channels can be measured by using the swelling assay, with liposomes reconstituted from purified porin and phospholipids (14, 15, 22). To get maximal photometric sensitivity, in our original method liposomes were made in 17% (wt/vol) dextran T-20 (containing 5 mM Tris-hydrochloride, pH 7.5) (14). Although these liposomes behaved in the expected way when diluted into nonelectrolyte solutions, they exhibited unexpected osmotic behavior towards electrolytes. For example, control liposomes without any porin were found to be isotonic with 35 mosM stachyose, whereas they showed rapid swelling in 35 mosM NaCl, and much higher concentrations of NaCl were required to get an apparent isotonic response. The most likely explanation is that the dextran preparation contains some charged groups (see reference 9). Alteration of ionic strength will then disturb the Donnan equilibrium and produce the unexpected osmotic response. The intravesicular accumulation of buffer cations via Donnan equilibrium also explains the fact that, although liposomes containing 17% (wt/vol) of 20,000-dalton dextran should be isotonic with $(170 \div 20{,}000) \times 1{,}000 = 9.5$ mosM nonelectrolytes, they are actually isotonic with 30 to 50 mosM sugars (14).

We therefore prepared the liposomes so that they contained 20 mM stachyose rather than dextran (and 5 mM Tris-hydrochloride buffer, pH 7.5). These liposomes, when made in the absence of added porin, behaved as predicted. Thus, the liposomes made in 20 mosM stachyose showed neither swelling nor shrinkage upon dilution into 20 mosM solutions of 1:1 or 2:1 electrolytes. However, when the liposomes were made with the addition of porin, dilution into an isotonic solution of sodium NAD resulted in a rapid swelling of only a short duration, even though the NAD anion was not expected to be able to penetrate rapidly through the porin channels due to its large size (663 daltons). Apparently, Na^+ ions flowed into the vesicles through the porin channels, driven by its high concentration gradient. This was accompanied by the influx of Cl^- ions, present in the Tris-hydrochloride buffer, against its own concentration gradient (in most experiments the initial concentrations of Cl^- were 2 mM both inside and outside the vesicles). The influx of NaCl resulted in the swelling of vesicles and continued until the driving force due to the unequal distribution of Na^+ across the membrane became matched by the driving force, in opposite direction, created by the unequal distribution of Cl^-.

These considerations indicated that buffers containing permeable anions could not be used for measurement of permeability of anions such as β-lactams. Swelling experiments were therefore carried out in solutions containing only impermeable anions, e.g., NAD, on both sides of the membrane. All solutions were made in freshly boiled distilled water to avoid the mass influx of bicarbonate anions. However, even with these precautions the presence of a large concentration gradient of Na^+ across the membrane often produced an unexpected osmotic behavior. For example, when the liposomes containing 10 mM sodium NAD were diluted into isotonic stachyose, there was a significant shrinkage, presumably caused by the efflux of Na^+ together with counterions, whose identity is not known. Furthermore, the presence of large concentration difference in Na^+ is expected to produce significant membrane potential due to the Donnan effect, and large membrane potential has been reported to result in the closure of porin channels, at least in a planar bilayer system (33, 34).

Ideally, therefore, the permeability of nonelectrolytes should be tested by using liposomes containing nonelectrolytes such as stachyose inside, and that of sodium salts should be tested by using liposomes containing sodium salts, such as sodium NAD, so that the creation of concentration gradients of sodium across the membrane could be avoided. In fact, we used this strategy in some of our experiments. However, one of our aims was to compare the penetration rates of electrolytes with those of nonelectrolytes, and turbidity changes of different liposome preparations could not be compared, as liposomes made in different media had different values of turbidity largely as a consequence of differences in average size and layer-to-layer distance. We therefore had to use a single preparation of liposomes for measuring the diffusion of nonelectrolytes, 1:1 electrolytes, and 2:1 electrolytes. Although it was possible to make liposomes and to dilute them in combinations of stachyose, sodium NAD, and the test solute so that the Na^+ concentration was always equal across the membranes, such a system had two drawbacks. (i) The concentration difference of the test solute across the membrane had to be kept at a low level. This decreased the driving force for diffusion and thus the sensitivity of the assay. (ii) Widely different external concentrations of NAD had to be used, depending on the nature of the test solute; this was not desirable because NAD in the external medium appeared

to inhibit slightly the influx of test solutes (data not shown).

In view of these results, the procedure described in Materials and Methods was devised as a compromise. In this system, the total external NAD concentration was always 2 mM, and thus the degree of inhibition of the solute influx was expected to be always identical. Furthermore, the concentration difference of the test solute across the membrane was kept at a constant and rather large value, 18 mosM. Finally, the concentration difference in diffusible cations across the membranes never exceeded threefold. Thus, the Donnan potential should have been <27 mV, according to the Nernst equation. Since 125 mV is reported to be necessary to close the channels in porin trimers (33), this very low potential was not expected to have much influence in permeability.

Diffusion of carbohydrates and their derivatives through the OmpF porin channel. The results obtained by the use of the liposome system described above are shown in Fig. 1a. First, the diffusion rates of nonelectrolytes are seen to decrease rapidly as the molecular size increases. This phenomenon was observed already with the porin from *E. coli* B (22), which appears to correspond to the OmpF porin of *E. coli* K-12 but may contain some differences in amino acid sequence (5, 32). This dependence is related to the diameter of the channel, and the similar solute size dependence (for example, disaccharides diffused at rates about two orders of magnitude slower than that of L-arabinose in both systems) suggests that the OmpF (K-12) channel is very similar in its diameter to the channel in the *E. coli* B/r porin.

Second, negatively charged compounds were found to diffuse through the channel significantly more slowly than the homologous, uncharged compounds. This is seen by comparing the rates for gluconate and glucuronate with that of glucose. The presence of two negatively charged groups further decreased the penetration rate, as seen in the comparison of saccharate and mucate with gluconate and glucuronate.

Diffusion through the OmpC channel. The diffusion process through the OmpC channel had characteristics similar to diffusion through the OmpF channel in its dependence on solute charge (Fig. 1b). Furthermore, by using intact cells we have shown that the hydrophobicity of the solute affected the penetration through the two channels in a very similar way (23).

However, we could detect two significant differences between the OmpC and OmpF channels. First, the dependence of diffusion rates on solute size appeared to be slightly more pronounced with the OmpC channel. For example, diacetamidodideoxyglucose penetrated at a rate only 1 to 2% of that of L-arabinose (Fig. 1b) through the OmpC channel, whereas its relative rate through the OmpF channel was about 5 to 6% (Fig. 1a). As the liposome filtration assay (18) is often more sensitive in detecting the "exclusion limits" of the pore, liposomes containing [^{14}C]dextran and ^{3}H-labeled oligosaccharides were made with OmpF or OmpC porins, and the retention of the ^{3}H-labeled sugars was compared after slow gel filtration of liposomes through columns of Sepharose 4B. There was much more pronounced retention of a trisaccharide, raffinose, in OmpC porin-containing vesicles even when twice as much OmpC protein was used in comparison with the OmpF protein (Table 1; see also below). Thus, the OmpC channel appears to be slightly narrower than the OmpF channel.

Another difference is in the magnitude of permeability produced by the addition of identical amounts of OmpF and OmpC porins. There was an approximately twofold difference in the permeability toward glucose (Fig. 2). This cannot be explained by the potential inactivation of OmpC porin during isolation, because a similar difference was seen when fragments of outer membranes, rather than purified porins, were used for reconstitution (data not shown). In fact, a similar difference was seen with intact cells (23). We feel that this difference in the "efficiency" is due to the difference in pore sizes between the OmpF and OmpC channels (see Discussion).

Diffusion through the PhoE channel. Figure 1c shows that the PhoE channel was similar to the OmpC channel in the diffusion of nonelectrolytes. Thus, the penetration rate decreased steeply with increasing size of the solute, and diacetamidodideoxyglucose diffused at 1% of the rate of arabinose. The apparent narrowness of the PhoE channel was also confirmed by the oligosaccharide retention assay (Table 1). However, lactose, with a higher molecular weight than diacetamidodideoxyglucose, diffused reproducibly more rapidly through the PhoE channel in the swelling assay (Fig. 1c), and this observation suggested that this channel could not be accurately described as a simple, nonselective channel. Indeed, the diffusion of monoanionic compounds was significantly accelerated in comparison with that of nonelectrolytes. The extent of acceleration varied depending on the size of the molecule, and lactobionate diffused nearly 10 times faster than lactose, although the penetration rates of gluconate and glucuronate were only about twice as high as that of glucose (Fig. 1c). The addition of a second negative charge appeared to reduce the permeation rate only slightly (cf. mucate and saccharate with gluconate and glucuronate in

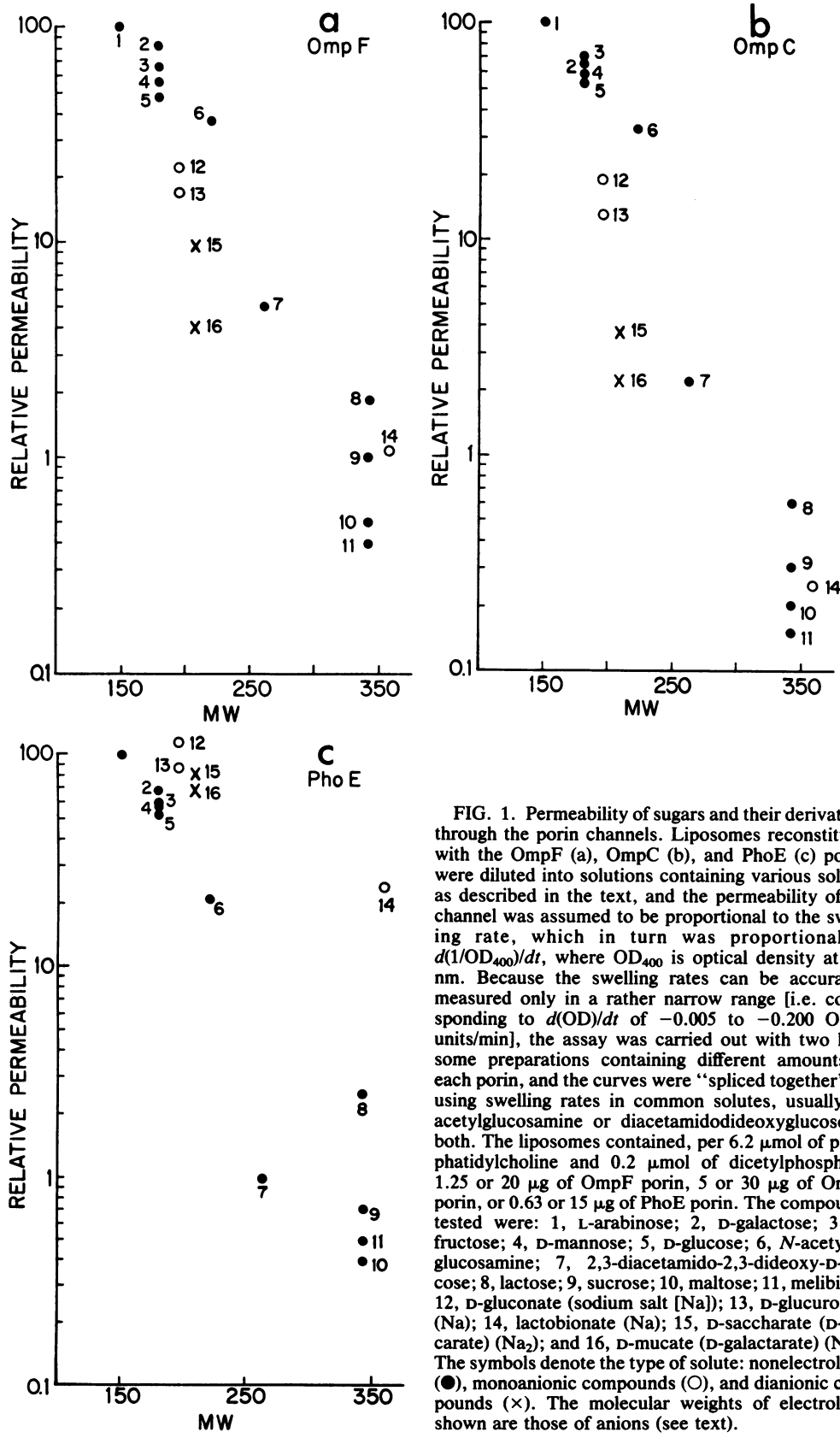

FIG. 1. Permeability of sugars and their derivatives through the porin channels. Liposomes reconstituted with the OmpF (a), OmpC (b), and PhoE (c) porins were diluted into solutions containing various solutes as described in the text, and the permeability of the channel was assumed to be proportional to the swelling rate, which in turn was proportional to $d(1/OD_{400})/dt$, where OD_{400} is optical density at 400 nm. Because the swelling rates can be accurately measured only in a rather narrow range [i.e. corresponding to $d(OD)/dt$ of -0.005 to -0.200 OD_{400} units/min], the assay was carried out with two liposome preparations containing different amounts of each porin, and the curves were "spliced together" by using swelling rates in common solutes, usually *N*-acetylglucosamine or diacetamidodideoxyglucose or both. The liposomes contained, per 6.2 μmol of phosphatidylcholine and 0.2 μmol of dicetylphosphate, 1.25 or 20 μg of OmpF porin, 5 or 30 μg of OmpC porin, or 0.63 or 15 μg of PhoE porin. The compounds tested were: 1, L-arabinose; 2, D-galactose; 3, D-fructose; 4, D-mannose; 5, D-glucose; 6, *N*-acetyl-D-glucosamine; 7, 2,3-diacetamido-2,3-dideoxy-D-glucose; 8, lactose; 9, sucrose; 10, maltose; 11, melibiose; 12, D-gluconate (sodium salt [Na]); 13, D-glucuronate (Na); 14, lactobionate (Na); 15, D-saccharate (D-glucarate) (Na_2); and 16, D-mucate (D-galactarate) (Na_2). The symbols denote the type of solute: nonelectrolytes (●), monoanionic compounds (○), and dianionic compounds (×). The molecular weights of electrolytes shown are those of anions (see text).

TABLE 1. Oligosaccharide retention assay of liposome permeability[a]

Porin added		Oligosaccharide diffused out during gel filtration (%)[b]	
Species	Amt (μg)	Sucrose	Raffinose
OmpF	30	97	78
OmpC	30	85	24
OmpC	60	97	45
PhoE	30	84	53

[a] *E. coli* phospholipids (1 μmol) and *E. coli* lipopolysaccharide (0.1 μmol) were mixed with the specified amounts of porin as described previously (18). The film was suspended in a buffer solution (0.2 ml) (18) containing approximately 4×10^5 cpm each of ^3H-labeled oligosaccharide and [^{14}C]dextran, the suspension was passed through a column of Sepharose 4B at a flow rate of 0.1 ml/min, and the liposome fraction was collected and counted.

[b] The $^3H/^{14}C$ ratios in the porin-containing liposomes were normalized by dividing by the $^3H/^{14}C$ ratio of the control liposomes. The percentage of ^3H-labeled oligosaccharide lost through efflux was calculated as 100 × (1 − normalized $^3H/^{14}C$ ratio).

Fig. 1c). The preference of the PhoE channel for compounds with negative charges was also seen in studies with intact cells (23).

Diffusion of some cephalosporins. To confirm the results obtained with intact cells (23), penetration rates of several cephalosporins were tested in the liposome system. In this experiment, however, one additional control had to be added. This was because more hydrophobic cephalosporins could cross the lipid bilayer regions of the membrane presumably in their protonated, uncharged form, followed by the influx of Na^+ through the porin channel. Thus, to estimate the contribution of this diffusion pathway, liposomes containing a cation channel, i.e., gramicidin channel, but without the porin channels were used. When swelling occurred upon dilution of these "gramicidin control" liposomes into a solution containing the sodium salt of a cephalosporin, this was evidence that the cephalosporin was penetrating through the lipid bilayer at a significant rate.

The results fully confirmed the conclusions drawn from the experiments with intact cells (Fig. 3). Thus, there was an inverse relationship between the hydrophobicity and permeation rate through porin channels among hydrophilic, monoanionic cephalosporins. The diffusion rates into liposomes, however, increased at the hydrophobic end of the spectrum, i.e., with cephalothin, cephaloram, and benzylpenicillin. That this was due to the increased penetration through the lipid bilayer was shown by high swelling rates of gramicidin control liposomes in solutions of these β-lactam compounds (Fig. 3).

In one experiment the diffusion rates of zwitterionic, anionic, and dianionic cephalosporins were compared by using liposomes reconstituted in 12 mM stachyose–4 mM sodium NAD–1 mM imidazole-NAD, pH 6.0 (see Materials and Methods). Table 2 confirms qualitatively the results obtained with intact cells. Thus, the OmpF and OmpC channels allowed the rapid diffusion of the anionic, hydrophilic cephacetrile and the zwitterionic cephaloridine, but the diffusion of the dibasic SCE-20 was considerably slower. However, the PhoE channel showed an entirely different behavior. It had a poor permeability toward large, uncharged (lactose) or zwitterionic (cephaloridine) solutes, but both monoanionic and dianionic compounds penetrated much more rapidly through this channel.

Diffusion of other solutes. We examined the rates of penetration of various peptides through OmpF and OmpC channels. They usually diffused with rates comparable to saccharides of similar sizes (Table 3), and there was no indication that the porin channels favored peptides with specific sequences or those of the L-series, again suggesting the absence of stereospecific recognition sites within the channels. The relative preference of each peptide for one channel over the other was also as predicted from the general properties of the channels. Thus, small (<190-dalton) peptides diffused two to three

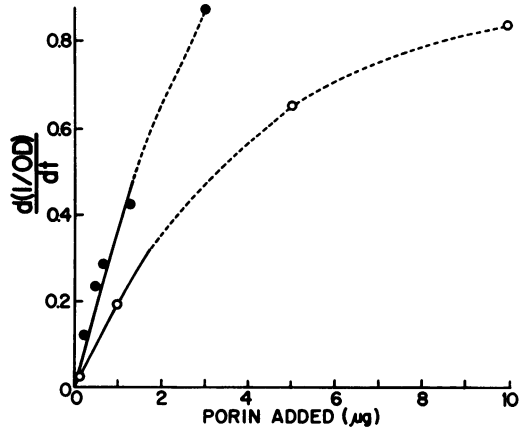

FIG. 2. Swelling rates of liposomes containing various amounts of OmpF (●) and OmpC (○) porins. Specified amounts of porin were added to a mixture of 6.2 μmol of phosphatidylcholine and 0.2 μmol of dicetylphosphate, the film was suspended in 0.4 ml of 12 mM stachyose–4 mM sodium NAD–1 mM imidazole-NAD (pH 6.0), and 20 μl of each suspension was diluted into 0.6 ml of 18 mM glucose–1 mM sodium NAD–1 mM imidazole-NAD (pH 6.0). The ordinate shows $d(1/OD)/dt$, where OD is optical density at 400 nm and t is in minutes. Under the conditions of these experiments, $d(OD)/dt$ of −0.23 OD unit/min corresponds to $d(1/OD)/dt$ of about 0.85.

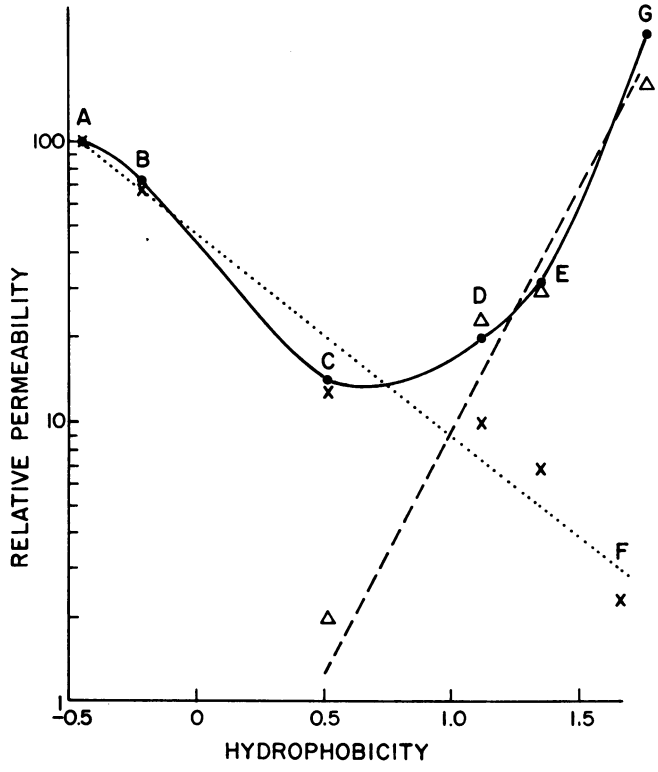

FIG. 3. Relative rates of permeation of monoanionic β-lactams. Liposomes containing 20 μg of OmpF porin in 6.2 μmol of egg phosphatidylcholine and 0.2 μmol of dicetylphosphate were made, as described in the text, in 7 mM sodium NAD–0.5 mM imidazole-NAD (pH 6.0) and were diluted into solutions containing 0.5 mM imidazole-NAD and isotonic concentrations of sodium salts of β-lactams. Hydrophobicity is expressed as the logarithm of the 1-octanol–water partition coefficient of the uncharged form of the solute (23); the value for compound G is from reference 3. Symbols: ●, initial swelling rates of porin-containing liposomes, relative to that in sodium cephacetrile; △, swelling rates of control liposome preparation, containing 1.5 μg of gramicidin A instead of porin, similarly diluted, and normalized to that of the experimental liposomes in sodium cephacetrile; ×, permeation rates determined in intact cells (23). The compounds used are: A, cephacetrile; B, cefazolin; C, cefamandole; D, cephalothin; E, cephaloram; F, benzothienylcephalosporin; and G, benzyl penicillin. Compound F could not be tested with liposomes because of its poor solubility. Compound G was not tested with intact cells because our β-lactamase assay did not detect the hydrolysis of penicillins.

times faster through the OmpF channel than through the OmpC channel, just as arabinose and glucose did (Table 3; Fig. 2). However, the difference between the rates through the two channels became more pronounced with peptides of higher molecular weight; this is presumably because the smaller size of the OmpC pore retards the penetration of these peptides more strongly (cf. trialanine, tetraalanine, and threonyl-leucine, Table 3). Interestingly, peptides that presumably could take the shape of a thin rod, such as *N*-acetyltetraglycine, diffused more rapidly than expected from their molecular weights and showed less discrimination between OmpF and OmpC channels.

It has been reported that *N*-formylmethionyl-leucyl-phenylalanine penetrated mainly through the OmpC channel (11). In the liposome system, this peptide diffused at about the same relative rate through OmpF- and OmpC-containing liposomes, but practically all of this diffusion took place through the lipid bilayer, as seen by the rapid swelling of gramicidin control liposomes in this peptide (Table 3). Thus, our results neither confirmed nor contradicted those of Heller and Wilson (11). However, these authors concluded that acidic peptides in general preferred the OmpC channel (11), and this conclusion was not borne out by our results (see experiment 2, Table 3). Furthermore, we could not repeat another aspect of the work of Heller and Wilson (11): they reported using a 20-mg/ml (i.e., 45.8 mM) solution of formylmethionyl-leucyl-phenylalanine, but in our hands the free acid of this peptide was essentially insoluble and even the sodium salt reached saturation at just below 11

TABLE 2. Swelling rates of liposomes containing various porins[a]

Solute	Charge	Initial swelling rate[b] of liposomes containing:		
		OmpF (20 μg)	OmpC (40 μg)	PhoE (20 μg)
Lactose	None	0.152	0.080	0.041
Lactobionate-Na^+	−	0.088	0.035	0.304
Cephaloridine	+ −	0.268	0.159	0.063
Cephacetrile-Na^+	−	0.119	0.059	0.226
SCE-20-Na_2^+	− −	0.023	0.011	0.105
5'-AMP-Na^+	−	0.048	0.018	0.118

[a] Liposomes were made, as described in the text, from a phosphatidylcholine-dicetylphosphate mixture and the porin indicated, and 20 μl of the suspension was diluted into 0.6-ml solutions containing 1 mM imidazole-NAD (pH 6.0)–1 mM sodium-NAD and 18 mM lactose (or cephaloridine), 9 mM lactobionate (or cephacetrile or 5'-AMP), or 6 mM SCE-20.

[b] Values are initial rate of decrease of optical density at 400 nm per minute.

mM, when the concentration was determined by the absorption of phenylalanine residue at 257 nm.

In another work (40), it has been reported that the OmpF protein formed a "specific" channel for nucleotides, including 5'-AMP. Although AMP diffused much faster through the OmpF channel than through the OmpC channel (Table 2), the difference was as predicted from the general properties of the channels, and there was no evidence for specificity.

DISCUSSION

Theoretical considerations on electrolyte diffusion. In this study we compared the swelling rates of liposomes upon dilution into 18 mM nonelectrolyte solutions as well as into 9 mM 1:1 electrolyte solutions. In addition to this difference in the molar concentrations of the diffusing solutes, with the electrolyte the intrinsic mobility of cations is usually different from that of anions. Thus, it becomes necessary for us to consider what the swelling rates mean in these tests.

If we denote by u, u_1, and u_2 the mobility of nonelectrolyte, cation, and anion, respectively, in crossing the membrane containing porin channels when driven by unit force, i.e., unit difference in solute concentration across the membrane, then the purpose of our experiments was to compare u of nonelectrolytes such as sugars with u_2 of such anions as monoanionic β-lactams and sugar acids. For nonelectrolytes, from the relationship between the permeability and diffusion coefficients

$$P = (k \cdot T/d) \cdot u \quad (1)$$

where P, k, T, and d denote permeability coefficient, Boltzman constant, absolute temperature, and the thickness of the membrane, respectively. For a 1:1 electrolyte, from equation 11.1 of reference 30,

$$P = (k \cdot T/d) \cdot 2\, u_1 \cdot u_2/(u_1 + u_2) \quad (2)$$

Now for the sodium salts of β-lactams or sugar acids, Na^+ is expected to have a much higher mobility, due to its smaller size and positive charge (2), than the bulky, negatively charged organic anions. (This assumption may not be

TABLE 3. Diffusion rates of peptides through porin channels[a]

Expt	Solute	Mol wt	Swelling rate of liposomes containing:	
			OmpF protein[b]	OmpC protein[c]
1	(L-Arabinose)	150	(1.0)	(1.0)
	(D-Glucose)	180	0.6	0.5
	L-Ala–L-Ala	160	1.1	0.9
	D-Ala–D-Ala	160	1.1	1.0
	L-Ser–L-Ala	176	0.9	0.7
	Gly-Gly-Gly	189	0.9	0.8
	L-Ser–L-Ser	192	1.0	0.8
	(*N*-Acetyl-D-glucosamine)	221	0.4	0.3
	L-Ala–L-Ala–L-Ala	231	0.7	0.3
	D-Ala–D-Ala–D-Ala	231	0.7	0.2
	L-Thr–L-Leu	232	0.6	0.1
	L-Lys–L-Asp	261	0.4	0.3
	L-Ala–L-Ala–L-Ala–L-Ala	302	0.3	0.1
2	(D-Gluconic acid [sodium salt])	195[d]	(1.0)	(1.0)
	Ac–L-Ala–L-Ala (sodium salt)	201	0.6	0.5
	Formyl–L-Met–L-Ala (sodium salt)	247	0.3	0.2
	Ac-Gly-Gly-Gly-Gly (sodium salt)	287	1.0	0.7
	Formyl–L-Met–L-Leu–L-Phe (sodium salt)	436	0.5[e]	0.5[e]

[a] Liposomes containing the specified porin were made by suspension in 20 mM stachyose–2mM imidazole-HCl, pH 6.0 (experiment 1), or in 7 mM sodium NAD–0.5 mM imidazole-NAD, pH 6.0 (experiment 2), and were diluted into isotonic solutions of test solutes containing the appropriate buffer. The swelling rates were normalized to those in L-arabinose (experiment 1) or sodium gluconate (experiment 2).

[b] In experiments 1 and 2, 1.0 and 2.0 μg of OmpF porin were added to 6.2 μmol of phospholipids and 0.2 μmol of dicetylphosphate, respectively.

[c] In experiments 1 and 2, 2.5 and 5.5 μg of OmpC porin were added, respectively.

[d] Molecular weights shown are those of the organic anions.

[e] Control liposomes containing 1.5 μg of gramicidin A instead of porin showed an exactly identical swelling rate in this solute.

correct for the PhoE channel [see below], and its permeability toward anions may be underestimated in this assay.) Thus, u_1 is much larger than u_2. Under these conditions, equation 2 simplifies to

$$P \simeq (kT/d) \cdot 2\, u_2 \qquad (3)$$

Thus, if a nonelectrolyte and an anion had identical mobilities through porin channels under a unit force, then the permeability coefficient for the latter will be twice as large as that for the former. Now, the swelling rate is proportional to the rate of influx (J) of solute (or ion) particles, which is given by $J = P \cdot A \cdot \Delta C$, where A is the area of the membrane and ΔC is the concentration difference of the solute across the membrane. If $u = u_2$, P for the anion is twice as high as the P for the nonelectrolyte, as we have seen. However, this difference disappears because ΔC for the 1:1 electrolyte (9 mM) was one-half of ΔC for the nonelectrolytes (18 mM); thus, the nonelectrolyte and the anion with the same mobility through the channel will give identical swelling rates. In other words, under our conditions the swelling rates are directly proportional to the mobility of nonelectrolytes and anions. Similar calculations showed that this conclusion could be extended to divalent anions.

Effect of solute parameters on penetration rates. The results presented here and in the accompanying paper (23) indicate that three gross physicochemical parameters of the solute molecule affect strongly its velocity of diffusion through porin channels. The effect of the size of the molecule has already been shown by using intact, growing cells of *E. coli* B/r (22), and the present study confirms the observation that even for molecules whose size is well within the exclusion limit of the pore, there is a drastic decrease in penetration rate as the molecular size increases. This is presumably not due to the presence of a collection of pores of different sizes, because at least one planar lipid film study, which can measure the conductivity caused by a single channel, showed that the pore size is rather uniform (33). This molecular size effect is therefore probably caused by collision of the solute molecule with the rims at the pore entrance and by the viscous drag exerted by the walls of the pore, as hypothesized by Renkin (29). The equation formulated by Renkin predicts that hollow cylindrical channels with radii of 0.58, 0.54, and 0.53 nm will produce the size dependence curve closest to the nonelectrolyte data points (Fig. 1) obtained with OmpF, OmpC, and PhoE porins, respectively. The significance of the absolute values of these figures is questionable, because it is uncertain whether the Renkin equation is applicable to these very narrow pores and because the shape of the pores may deviate strongly from the hollow cylinder. However, the data at least suggest the relative sizes of channels produced by the three porins.

The effect of electrical charges again confirmed and extended our studies with intact cells (23). In the experiments with intact cells, we were limited mostly to comparisons between the permeability of a real β-lactam compound and the expected permeability calculated for a hypothetical compound, because pairs of compounds differing only in electrical charges were not available. Furthermore, uncharged β-lactam compounds which were soluble enough in water and susceptible enough to β-lactamase hydrolysis could not be found, and our assessment of the effect of negative charge had to be based on the comparison of anionic compounds with zwitterionic compounds rather than with uncharged analogs. In a similar vein, although the use of planar lipid films allowed the comparison of anion versus cation penetration rates (2), the rates for uncharged molecules could not be measured easily with this technique. It is thus gratifying to see that the introduction of a single negative charge into a nonelectrolyte decreased the rate of diffusion of molecules of approximately 200 daltons through the OmpF and OmpC channels by a factor of 3 (Fig. 1a and b; cf. gluconate and glucuronate with glucose). As we have seen with intact cells (23), the presence of a second negative charge further decreased the rates of diffusion through OmpF and OmpC channels (Fig. 1a and b).

In striking contrast, the permeation rates through the PhoE channel became much higher when a negatively charged group was added to a nonelectrolyte and did not decrease much even when a second anionic group was added (Fig. 1c). In fact, with larger solutes such as lactobionate, the acceleration over the diffusion rate of its nonelectrolyte analog, lactose, was so great that the pore behaved essentially as an "anion-specific" channel. The behavior of the PhoE channel is similar to that of another specific channel, i.e., the LamB channel produced by the phage lambda receptor protein of *E. coli* (14, 15). Thus, in both cases the channels act as rather stringent, selective channels for larger solutes, yet at the same time they act as nonspecific pores for smaller solutes; in fact, 1 μg of PhoE and OmpF proteins produced almost the same swelling rates in arabinose, a result confirming that the PhoE pore functions as a highly efficient pore for small nonelectrolytes. The preference for anions obviously is in agreement with the idea that the physiological function of PhoE channel may be the transport of molecules carrying multiple negative charges, such as phosphate and phosphorylated compounds, as suggested by the derepression of PhoE porin

upon phosphate starvation (1, 26). In our study, however, we could not observe any preference for the phosphate group over other anionic groups (Fig. 1c; Table 2). Perhaps the channel simply prefers any negatively charged compounds; alternatively, more refined analysis may be necessary to reveal the specific interaction between the solutes and the channel.

Although the effects of electrical charges on the rates of permeation of β-lactams were qualitatively similar in liposomes and in intact cells, interesting quantitative differences were observed between the two systems (Table 2). In the liposome system, the diffusion rate of the anionic cephacetrile was about one-half to one-third of the zwitterionic cephaloridine, through both OmpF and OmpC channels. In contrast, in intact cells, cephacetrile diffuses three to seven times more slowly than cephaloridine through these channels (23). As another example, whereas the divalent SCE-20 anion penetrated through OmpF and OmpC channels at about one-tenth of the rate of cephacetrile in liposomes, the differences became much larger, up to 25 to 40 times in intact cells (see Fig. 3 and Table 2 of reference 23). These differences could be due to the presence of membrane potential (interior negative) across the outer membrane in intact cells (35); such a potential would further decrease the rate of diffusion of anionic (and even more strongly that of multiply anionic) compounds beyond the discrimination produced by the channel itself.

Finally, our liposome study (Fig. 3) confirmed the effect of hydrophobicity observed in intact cells (23). This agreement indicated that the major portion of the more hydrophilic cephalosporins cross the *E. coli* outer membrane via porin channels, in confirmation of the finding that the penetration rates of these antibiotics were drastically reduced in porin-deficient mutants (24). However, the uncorrected penetration rates in liposome experiments showed a biphasic rising limb with more hydrophobic β-lactams (Fig. 3), which were shown, through the use of gramicidin control liposomes, to penetrate predominantly through the lipid bilayer region of the membrane. The absence of this rising limb in the intact cell permeation experiments shows that the lipid domains of outer membranes are unusually resistant to penetration by hydrophobic solutes, as was inferred earlier (19).

Porins and bacterial physiology. Because the *E. coli* porin channel is quite narrow, the permeation of larger, more hydrophobic, or negatively charged compounds is slowed down considerably. Furthermore, the diffusion rate of any given solute across the outer membrane decreases essentially in proportion to its external concentration, when the concentration is low. Thus, the outer membrane becomes a significant permeation barrier, for example, for the uptake of maltose at concentrations below 10^{-4} M, as shown by calculations in reference 22. The production of a maltose-specific channel, phage lambda receptor (LamB) protein (14, 15, 20), presumably accelerates the influx of maltose. This idea, however, did not fit well with the data of another laboratory (36), claiming that LamB and porin channels are permeable to maltose to approximately the same extent. In contrast, the swelling assay performed under our standard conditions showed that the permeability of the LamB channel to maltose was at least 25 times higher than that of the OmpF channel (data not shown), as expected from the physiological considerations.

The presence of multiple species of porins in a single organism, and the observation that the production (or incorporation) of each of them is controlled by the growth conditions (for review, see reference 21) suggested different physiological roles for each of these porins. The PhoE protein indeed appears to play a unique role in the uptake of phosphate or phosphorylated compounds and to have some solute specificity, although at the same time it allows the nonspecific diffusion of nonphosphorylated compounds. For the two porins produced in ordinary media, i.e., OmpF and OmpC porins, amino acids (17, 28), sugars of a small size (<200 daltons) (28), and β-lactams (39) were reported to pass through either of these channels. In contrast, nucleotides such as 5'-AMP (28, 40, 41), tetracycline (6, 27), and chloramphenicol (27) were reported to penetrate predominantly through the OmpF channel, and an anionic peptide was claimed to diffuse predominantly through the OmpC channel (11). Some workers interpreted these results to be evidence for the presence of specificity in the porin channel (11, 40).

Our study, in contrast, failed to reveal any evidence for true specificity in OmpF and OmpC channels (Fig. 1; Table 3) and instead showed that all solutes (including anionic peptides) diffused faster through the OmpF channel than the OmpC channel, the rate difference being about twofold for such small solutes as arabinose or glucose (Fig. 2) and increasing to tenfold (and possibly even more) for larger solutes such as lactose (Table 2), larger peptides (Table 3), and β-lactams (Table 2; Fig. 3; Table 2 of reference 23). We believe that our results as well as most of the pore preference data from other laboratories can be simply explained by the slightly smaller equivalent pore radius of the OmpC channel. The use of the Renkin equation (29) predicts that the pore with a 0.54-nm radius (OmpC) will have 62% of the permeability of the

pore with a 0.58-nm radius (OmpF) for a solute with a Stokes radius of 0.42 nm, such as glucose. This is close to the difference observed in reconstituted liposomes (Fig. 2). The discrimination based on the difference in pore size will obviously become more pronounced for larger solutes. This is what we have found, and it explains why large molecules such as 5'-AMP or tetracycline predominantly diffuse through the OmpF channel. With sugars and amino acids (17, 28), the criterion for the detection of pore preference was whether the overall rate of active transport was visibly decreased in mutants lacking one of the porins. Since these small solutes diffuse rapidly through both OmpF and OmpC pores, it is not surprising that not much decrease was noticed in mutants producing only OmpC protein. At present, therefore, there is no necessity to postulate that these channels are specific or that different fractions of the populations of these channels are in a "closed" conformation. We could not confirm the claimed specificity of the OmpC channel toward anionic peptides (11) (Table 3); our results are also at variance with those of van Alphen et al. (39), who reported that a large fraction of cephaloridine and ampicillin apparently diffused through the OmpC channel.

The OmpC channel can therefore be thought of as a more stringent, more discriminating channel than the OmpF pore. The coexistence of these two types of channels may give some ecological advantages to *E. coli*. When *E. coli* K-12 was exposed to β-lactam antibiotics that penetrated slowly through the porin channels, mutants producing only the OmpC porin were selected for (10). Since the OmpC channel has probably only 15 to 20% of the permeability of the OmpF channel for these compounds (see Table 2 of reference 23 and Table 2, this work), but retains as much as 50% of the OmpF channel permeability for small essential nutrients such as glucose, we can see that the generation of mutants of this type will allow the survival of the *E. coli* population in the presence of noxious compounds by drastically reducing their influx rate, yet at the same time allowing the adequate influx of nutrients.

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ADDENDUM IN PROOF

After this paper was accepted for publication, we found that the paper of R. Benz and R. E. W. Hancock (Biochim. Biophys. Acta **646**:298–308, 1981) contained a table that includes the conductivity data on *E. coli* K-12 porins, quoted as unpublished data of R. Benz and U. Henning. The data indicate that the conductivity of the OmpF channel is slightly higher, by 4%, than that of the OmpC channel, but there is no comment on the statistical or biological significance of the observed difference. We have also been informed by Taiji Nakae that he also found that the OmpF channel was larger than the OmpC channel.

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