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Diffusion of β-Lactam Antibiotics Through the Porin Channels of *Escherichia coli* K-12

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Diffusion rates of various β-lactam antibiotics through the OmpF and OmpC porin channels of *Escherichia coli* K-12 were measured by the use of reconstituted proteoliposomes. The results can be interpreted on the basis of the gross physicochemical properties of the antibiotics along the following lines. (i) As noted previously (Nikaido et al., J. Bacteriol., 153:232–240, 1983), there was a monotonous dependence of the penetration rate on the hydrophobicity of the molecule among the classical monoanionic β-lactams, and a 10-fold increase in the octanol-water partition coefficient of the uncharged molecule decreased the penetration rate by a factor of 5 to 6. (ii) Compounds with exceptionally bulky side chains, such as mezlocillin, piperacillin, and cefoperazone, showed much slower penetration rates than expected from their hydrophobicity. (iii) The substituted oxime side chain on the α-carbon of the substituent group at position 7 of the cephem nucleus decreased the penetration rate almost by an order of magnitude; this appears to be largely due to the steric effect. (iv) The presence of a methoxy group at position 7 of the cephalosporins also reduced the penetration rate by 20%, probably also due to the steric hindrance. (v) Zwitterionic compounds penetrated very rapidly, and the correlation between the rate and hydrophobicity appeared to be much weaker than with the monoanionic compounds. Imipenem showed the highest permeability among the compounds tested, presumably due, at least in part, to its compact molecular structure. (vi) Compounds with two negative charges penetrated more slowly than did analogs with only one negatively charged group. Among them, only moxalactam, ceftriaxone, and azthreonam showed penetration rates corresponding to, or higher than, 10% of that of imipenem.

β-Lactam antibiotics are currently used widely in the treatment of infections caused by gram-negative bacteria. These bacteria are surrounded by an outer membrane which contains porin proteins that produce transmembrane diffusion channels (15, 16, 19). It has been previously shown (3, 22) that, by using mutants producing greatly decreased amounts of porins, cephaloridine and 6-aminopenicillanic acid diffuse across the *Escherichia coli* outer membrane primarily through the porin channel, and this finding has been confirmed since then for many other β-lactam antibiotics, including ampicillin, benzylpenicillin, cephacetrile, cefamandole, and cephalothin (H. Nikaido and E. Y. Rosenberg, unpublished data). This was also confirmed in *Enterobacter cloacae* and *Proteus mirabilis* by using mutants lacking what appeared to be porins (24). Thus, most of the β-lactam antibiotics appear to penetrate the outer membrane of both *E. coli* and other enteric bacteria (and probably many other gram-negative bacteria) through porin channels, and it becomes important to understand the interaction between β-lactams and the porin channel.

We have previously examined the rates of diffusion of several β-lactam compounds through *E. coli* porin channels both in intact cells (21) and in reconstituted proteoliposomes (20). The primary purpose of these studies, however, was to examine the properties of the porin channel per se; therefore, we used only a small number of β-lactams selected on the basis of their physicochemical properties rather than on the basis of their chemotherapeutic utility. We thought, therefore, that a more comprehensive survey of compounds of a wider range would be useful, especially now that new "third generation" agents not used in our previous studies are currently in use in clinical applications. This study presents such a survey, carried out by using the reconstituted proteoliposome system. *E. coli* K-12 produces in ordinary culture media two porins, OmpF and OmpC, which seem to be different only in the apparent size of the channel (20). In this study, we emphasized the OmpF porin, the porin with the wider pore diameter, and then compared the results with those obtained with less extensive studies with the OmpC porin. We expect that the conclusions obtained in this study are also valid, at least in a qualitative sense, with porins of many other gram-negative organisms.

MATERIALS AND METHODS

Chemicals. The sources of most chemicals are given in references 20 and 21. The sources of β-lactam compounds are given parenthetically as follows: cefazolin, carbenicillin, ampicillin, and benzylpenicillin (Sigma Chemical Co., St. Louis, Mo.); cephacetrile (CIBA-Geigy, Basel, Switzerland); cefmetazole and its 7-α-H analog (S. Sugawara, Sankyo Co. Ltd, Tokyo, Japan); cefotaxime and its *anti-* analog, R02-5328A, as well as the *S*-oxide analog of cefotaxime, HR109, and its *anti-* analog, S810592 (E. Schrinner, Hoechst, Frankfurt-am-Main, Federal Republic of Germany); cyclacillin (Wyeth Laboratories, Philadelphia, Pa.); penicillin N (Abbott Laboratories, North Chicago, Ill.); cefotiam, cefsulodin, sulbenicillin, SCE-20, and SCE-796 (Takeda Chemical Co., Osaka, Japan); ceforanide, cephapirin, MR-S93, MR-S94, and BL-S217 (R. Morin, Bristol-Myers Co., Syracuse, N.Y.) imipenem (MK-0787) and cefoxitin (Merck, Sharpe & Dohme Research Laboratories, Rahway, N.J.), ceftizoxime, nocardicin A, and nocardicin B (Fujisawa Pharmaceutical Co., Osaka, Japan); azthreonam (R. B. Sykes, Squibb Institute for Medical Research, Princeton, N.J.); cefuroxime and ceftazidime (Glaxo Research Group, Greenford, Middlesex, United Kingdom); ceftriaxone (Roche Di-

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agnostics, Div. Hoffman-La Roche, Inc., Nutley, N.J.); cefoperazone (Pfizer Inc., New York, N.Y.); azlocillin and mezlocillin (Bayer AG, Leverkusen, Federal Republic of Germany); cephalexin, cefaclor, cephamandole, cefaparole, cephaloglycin, moxalactam, cephalosporin C, LY097964, and LY151884 (Eli Lilly & Co., Indianapolis, Ind.); and piperacillin (Lederle Laboratories, Pearl River, N.Y.).

Determination of hydrophobicity. As in our earlier work, the hydrophobicity of the compounds was determined as the 1-octanol–water partition coefficient of the uncharged form of the molecule (P_u) (21). P_u values were determined experimentally or calculated as previously described (21), starting from the P_u values of certain compounds determined experimentally. For some substituents, the ab initio calculation of their fragmental constants was nearly impossible; fortunately, we had compounds which allowed the experimental determination of P_u in most cases. Thus, cephamandole served as a starting point for calculations for compounds containing tetrazole side chains; for compounds containing substituted oxime structure, cefuroxime was used as the starting point (see Table 1). For sulbenicillin and cefsulodin, the fragmental constant of Hansch and Leo (8) for an SO_3 group was used.

Liposome swelling assay. OmpF and OmpC porins were purified from *E. coli* K-12 strains JF701 and JF703 as previously described (20). *Brucella abortus* 19 porin has also been previously described (5). Since most β-lactam compounds showed rather low permeability through the porin channels, it was necessary to use large amounts, up to 50 μg of porin protein, for reconstitution with 6.2 μmol of phosphatidylcholine and 0.2 μmol of dicetylphosphate (17, 20). The liposomes were resuspended in a mixture of 12 mM stachyose–4 mM sodium NAD–1 mM imidazole NAD buffer (pH 6.0) and were diluted in isotonic solutions of β-lactams. For zwitterionic, monoanionic, and doubly anionic compounds, the isotonic concentrations were close to those described previously (20) in solutions containing 1 mM sodium NAD and 1 mM imidazole NAD (pH 6.0) (20). This minimized the creation of membrane potential and the potential-induced movement of ions. To make the results of various experiments comparable to each other, the results were normalized to the permeation rate of cephacetrile, which was assigned the value of 100 (20).

The β-lactam stock solutions were made at the concentration of 25 mM in 1 mM sodium NAD–1 mM imidazole NAD (pH 6.0). The pH of the solution was then carefully adjusted to 5.8 to 6.2 with NaOH. When the compound was supplied as sodium salt, pH adjustment required only trace amounts of NaOH. However, with zwitterionic compounds, up to 0.15 eq of NaOH was sometimes required, and with free acid forms of mono- and dianionic compounds, nearly theoretical amounts of NaOH had to be added. It was necessary to pay close attention to the preparation of solutions, as anions with high permeability through porin channels produced very rapid swelling that had little relation to the penetration rates of β-lactams. For this reason, we have not been able to determine the penetration rates of cefotiam and cefmenoxime which were supplied as chloride salts. Some other agents could not be studied because they contained extraneous small molecules; an example is cefatrizine, which was supplied as the propyleneglycol soluate. When acidic compounds were supplied as salts of elements other than sodium, they had to be converted to sodium salt as previously described (20). However, with sodium and other salts of zwitterionic compounds, removal of the cation was difficult; therefore, these compounds could not be studied. Cephapirin, which was supplied as a sodium salt, and ceforanide, which was supplied as a lysine salt, belonged to this class. Many compounds could not be studied because of insufficient solubility; these include cefaparole, MR-S93, and MR-S94. We also found that very different swelling rates were observed when the pH of the sample deviated significantly from 6.0.

It was essential to make the solution isotonic with the liposomes. Since β-lactams often contained unspecified amounts of water, the concentrations of their solutions were measured by using as an osmometer the liposomes that did not contain porins. For example, when the liposomes showed slight shrinkage when diluted into 21 mM stachyose, they were then tested with 20.5 mM stachyose, 20 mM stachyose, etc., until the concentration producing neither shrinkage nor swelling was found. If the same batch of liposomes showed isotonic behavior with 20 mM stachyose and also with the solution of cephacetrile that had a nominal concentration of 11.5 mM, these two solutions are obviously isotonic with each other. Unfortunately, however, these concentrations could not be used directly in the experiment with porin-containing liposomes, for these liposomes displayed, for unknown reasons, isotonic concentrations usually slightly lower than those of non-porin-containing liposomes. Thus, if the porin-containing liposomes showed neither swelling nor shrinkage upon dilution into 19 mM stachyose, then cephacetrile was used in an $11.5 \times (19/20) = 10.9$ mM nominal concentration with the porin-containing liposomes. It is extremely important to follow the details of these procedures for the adjustment of solute concentrations.

A major problem with this assay was that hydrophobic β-lactams, especially monoanionic compounds, gave spuriously high values, apparently because the diffusion of the uncharged form through the lipid bilayer was accompanied by the influx of counter ions through the porin channel. To detect the occurrence of this phenomenon, we routinely ran a control with liposomes containing gramicidin A (Sigma) instead of porin and discarded all data on that particular antibiotic when the gramicidin control showed significant rates of swelling (20). This limited us to examining more hydrophilic antibiotics, especially among monoanionic compounds. In terms of log P_u, we were essentially limited to compounds with log P_u values of less than 0.6. However, with zwitterionic and doubly negatively charged compounds, the diffusion through the lipid bilayer was much slower, and this difficulty was rarely encountered, regardless of the values of the calculated P_u.

RESULTS AND DISCUSSION

Results of swelling assay. Table 1 shows the complete set of results on the rates of penetration of various β-lactams through the OmpF and OmpC porin channels, and Fig. 1 shows the structure of most of the compounds tested. The assay was based on the initial rate of swelling of liposomes, a parameter that could be influenced by small amounts of impurities present in the preparation, especially when the β-lactam had low rates of penetration. To assess the relevance of this factor, we routinely calculated the ratio of initial rates of swelling to the rates of swelling at the 80- to 100-s interval, when initial swelling or shrinkage due to minor impurity was likely to have ended. For most compounds, the ratio was ca. 4.6, indicating that the curves had a similar shape, regardless of the magnitude of the initial diffusion rate. However, for piperacillin, carbenicillin, sulbenicillin, and ceftazidime, the ratios were much higher than 4.6, indicating that the initial swelling was due to the

TABLE 1. Relative permeation rates of β-lactam antibiotics[a]

β-Lactam	Mol wt[b]	Hydrophobicity (log P_u)[c]	Relative diffusion rate[d] through:	
			OmpF channel	OmpC channel
Monoanionic compounds				
Cephacetrile	338	−0.45	100	100
7-α-H-cefmetazole	440	−0.62	82 (72–95)	
Cefazolin	453	−0.24	77 (74–81)	
Cefmetazole	470	−0.60	65 (63–68)	
S810592	470		63 (57–69)	
R02-5328A	454	(−1.05)[e]	52 (43–60)	
Cefoxitin	426	−0.02	46 (44–48)	
LY151884	396	(−0.30)[e]	41 (37–45)	
Ceftizoxime	382	(−0.80)[e]	35 (32–40)	19
HR109	470		30 (24–34)	
Cefotaxime	454	(−1.05)[e]	22 (20–25)	8
LY097964	396	(−0.30)[e]	22 (21–24)	
Cefoperazone	644	−0.74	16 (14–19)	
Cefamandole	461	0.50	14[f]	
Cefuroxime	410	−0.16	7 (3–11)	
Piperacillin	516	0.50	<5	
Zwitterionic compounds				
Imipenem	299	(−1.94)[g,h]	216 (200–228)	280
Cephaloridine	415	(2.04)[i]	167 (150–195)	
Cephalexin	347	(1.28)[i]	129 (118–140)	
Cefaclor	368	(1.10)[i]	123 (98–150)	
Cephaloglycin	405	(0.53)[i]	87 (78–99)	
BL-S217	437	(1.50)[i]	84 (75–98)	
Ampicillin	333	(0.95)[g]	46 (43–49)	
Cyclacillin	341	(1.96)[g]	40 (35–44)	
Dianionic compounds				
Moxalactam	518	(−2.86)[i]	34 (29–38)	10
Azthreonam	433		22 (19–25)	12
Ceftriaxone	552		20 (16–25)	<4
Sulbenicillin	412	(0.42)[g]	5 (3–8)	
Carbenicillin	376	(1.38)[g]	5 (2–8)	
Compounds with one positive and two negative charges:				
Cephalosporin C	414	(−0.88)[i]	72 (65–83)	
Penicillin N	358	(−0.46)[g]	56 (46–65)	
Cefsulodin	531	(−0.07)[i]	37 (24–46)	
SCE-796	495	(0.89)[i]	34 (33–36)	
Nocardicin B	499		33 (28–40)	
Nocardicin A	499		23 (18-25)	
Ceftazidime	545	(0.75)[e]	12 (10–13)	<4

[a] The data show the rate of diffusion of β-lactam molecules through the OmpF and OmpC porin channels in the presence of an identical driving force. For OmpF, an average of three or more experiments, performed with three separate porin preparations over the span of 3 years, is shown, and the range of values obtained are listed in parentheses. Mezlocillin and azlocillin gave low swelling rates (10 to 15% that of cephacetrile) in OmpF liposomes, but they are not listed because gramicidin control (see the text) showed very slow swelling with these compounds. For OmpC, average values from three swelling assays performed with a single proteoliposome preparation are shown.

[b] The molecular weights of compounds with net anionic charge(s) are those of free anions.

[c] Hydrophobicity values without parentheses were experimentally determined as described previously (21). Those in parentheses were calculated (21).

[d] The rates were normalized to the swelling rate in cephacetrile. The actual rate of swelling of OmpC-containing proteoliposomes in cephacetrile was 25 to 30% of the rate obtained in proteoliposomes containing the same amount of OmpF porin (20).

[e] Calculated by using the experimentally determined P_u of cefuroxime as the starting point.

[f] From reference 20.

[g] Calculated by using the experimentally determined P_u of benzylpenicillin as the starting point.

[h] The accuracy of this value may be low because the structure deviates extensively from that of benzylpenicillin.

[i] Calculated by using the experimentally determined P_u of cephaloram.

presence of minor impurities. The penetration rates of these compounds were therefore calculated on the basis of 80- to 100-s swelling rates. S810592 and R02-5328A, on the other hand, showed ratios much lower than 4.6, suggesting the presence of impurities producing transient shrinkage of liposomes. Their penetration rates, therefore, were also calculated on the basis of 80- to 100-s rates and are thus approximate values.

It should be mentioned that the swelling assay detects the flux of any ions and compounds; this assay, therefore, must be used with extreme care, especially when the penetration of charged solutes is studied. The use of dextran-containing liposomes for the assay of β-lactam diffusion or the use of such liposomes for measuring the penetration of positively charged solutes such as aminoglycosides do not produce meaningful results, as buffer ions show complex flux patterns in and out of liposomes in these situations. Some workers do not adjust the solutes for isotonic conentrations and wait until the initial phase of swelling or shrinkage is over (14). This strategy, which works well with systems

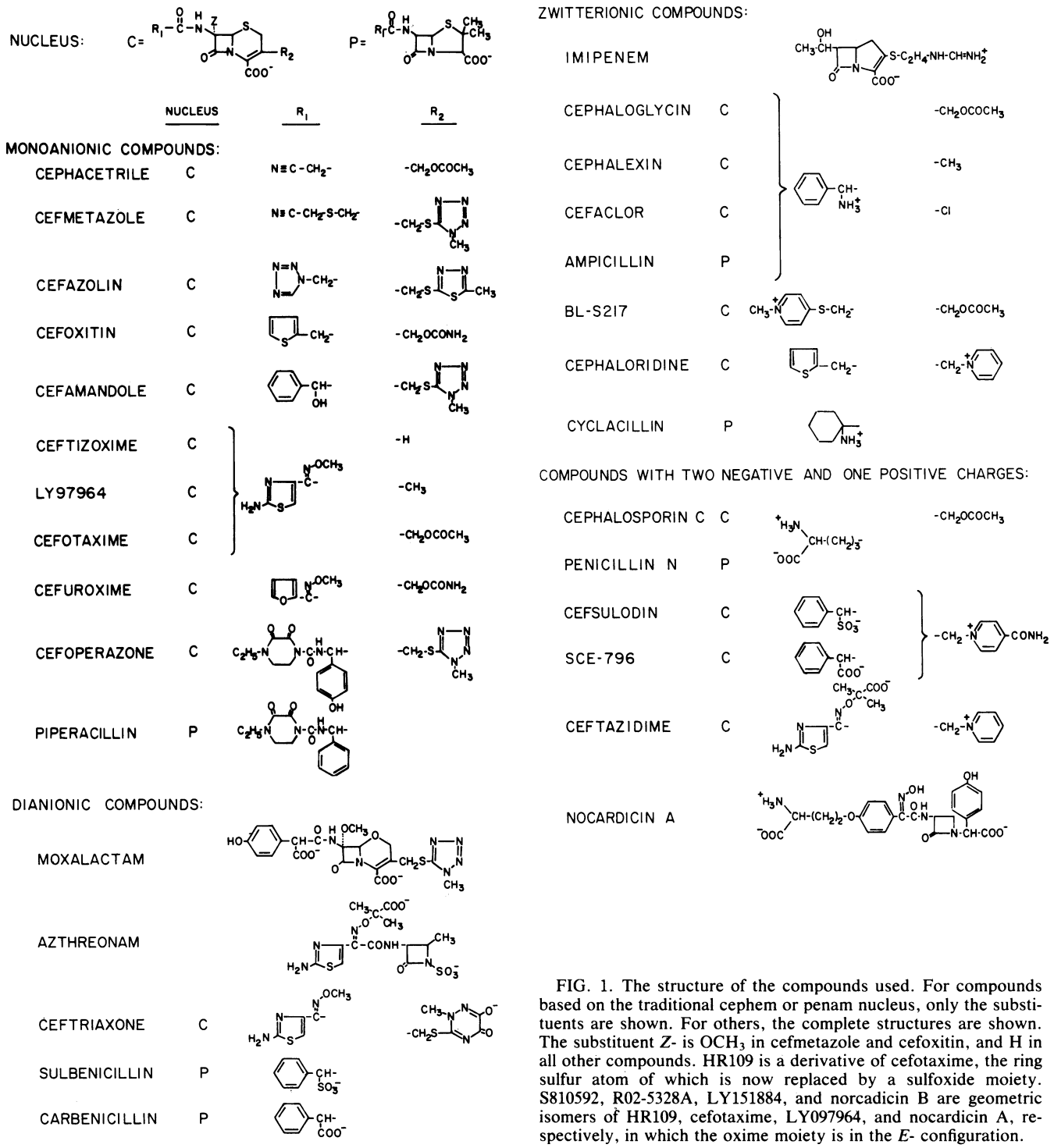

FIG. 1. The structure of the compounds used. For compounds based on the traditional cephem or penam nucleus, only the substituents are shown. For others, the complete structures are shown. The substituent *Z*- is OCH_3 in cefmetazole and cefoxitin, and H in all other compounds. HR109 is a derivative of cefotaxime, the ring sulfur atom of which is now replaced by a sulfoxide moiety. S810592, R02-5328A, LY151884, and norcadicin B are geometric isomers of HR109, cefotaxime, LY097964, and nocardicin A, respectively, in which the oxime moiety is in the *E*- configuration.

with single membrane layers (such as erythrocytes [26]), unfortunately does not work with multilayered liposomes, because at the point at which no flux is apparent, the shrinkage of outer layers may be being balanced by the swelling of the inner layers. In our system, however, the swelling rates were directly proportional to the permeability of β-lactams. Although 0, 50, and 67% of the swelling were due to the influx of counter ions when zwitterionic, monoanionic, and dianionic compounds, respectively, were used, this difference was compensated for by the difference in the concentration of these compounds used, as described previously (20).

For most compounds, the assay was repeated at least four times. As discussed previously (20), these data show the relative rate of permeation of various β-lactams when a fixed driving force (e.g., a concentration difference across the outer membrane of 10 μM) exists across the given membrane containing OmpF porin. The actual rate can be altered in at least two ways. (i) If the driving force is altered, e.g., by doubling the concentration outside, the rate of influx will

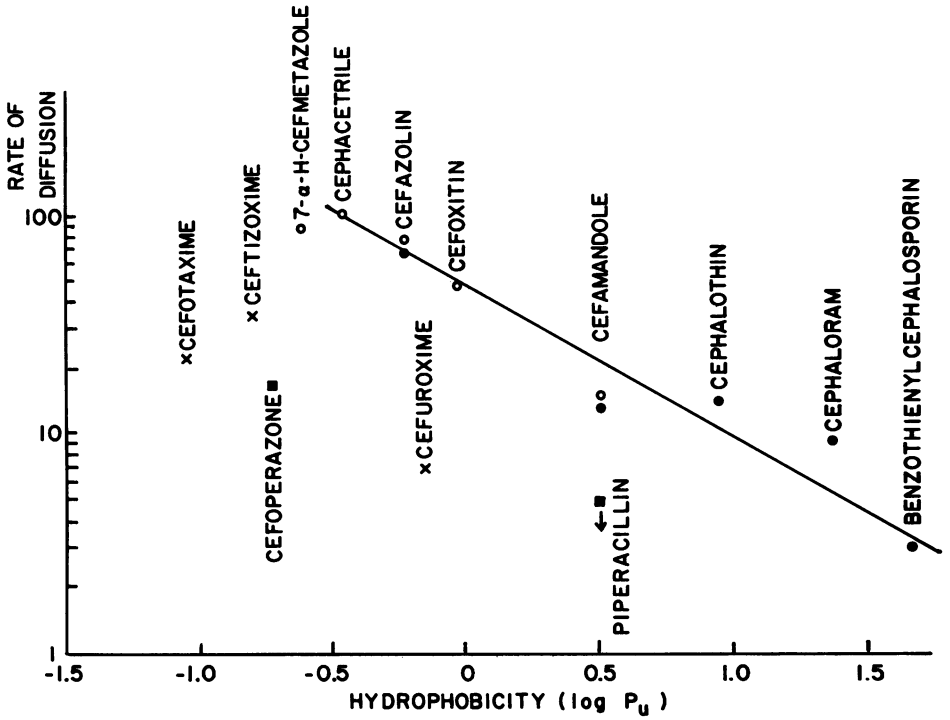

FIG. 2. The permeability of monoanionic cephalosporins. The rate of penetration through the OmpF channel is expressed by assigning the value of 100 to the rate for cephacetrile. The rates for "conventional" cephalosporins determined by the liposome assay (○) are complemented with those determined by the use of whole cells (●) (21), because the former assay is not usable for more hydrophobic compounds (20). The rates determined by the liposome swelling assay for compounds with methoxime substituents (×) and for compounds with exceptionally bulky side chains (■) are also shown. Hydrophobicity is expressed as log P_u (see the text).

also be doubled. (ii) The porin content of the membrane also influences the actual rate. Thus, if the porin content of the outer membrane is decreased by a factor of 10, this will decrease the actual rate of diffusion of all compounds by a factor of 10.

As pointed out above, it is not possible to devise a system that does not create membrane potentials or unequal distributions of some ions and yet is capable of assaying the permeation of molecules carrying various numbers of net negative charges. Thus, our system is a compromise, and the results, especially the values for slowly penetrating compounds, should be considered only as approximate values. Another complication is the use of NAD as the "channel-impermeable" anion. This method was developed for liposomes containing relatively small amounts (usually 1 to 5 μg of porin per 6.2 μmol of phospholipids) of porin (17, 20); in these liposomes, NAD behaved as though it was essentially impermeable. However, the vesicles used in this investigation contained up to 50 μg of porin so that visible swelling was produced even with the slower-penetrating compounds; it was thus found that in this system NAD penetrated through the channel, especially the OmpF channel, with significant rates. (This is not surprising since *E. coli* and *Salmonella typhimurium* are known to utilize the exogenous NAD for biosynthesis [6]). In our system, the interference due to the movement of NAD was kept to a minimum, presumably because the inside high concentration gradient of NAD was balanced, more or less, by the outside high concentration gradient of the counter ion Na^+, at least in the assays with anionic β-lactams. Nevertheless, until a less permeable anion is found and used in this assay, the rates of penetration of slowly penetrating compounds should be considered approximate and tentative for this reason also.

Interesting comparisons can be made among various subsets of these compounds, and we describe first the extensive set of results obtained by using proteoliposomes containing the OmpF porin.

Effect of hydrophobicity in monoanionic compounds. As described earlier, there is a rather strict relationship between the hydrophobicity and the magnitude of the permeability coefficient among the "classical" monoanionic β-lactams (20, 21). This relationship was also confirmed in this study, and the 7-α-H analog of cefmetazole, cefmetazole, and cefoxitin behaved more or less as expected from their hydrophobicity values (Fig. 2); with cefmetazole and its analog, however, the diffusion rates were slightly slower than expected, possibly as a consequence of the presence of a rather bulky methyltetrazole substituent at C-3.

Effect of bulky or protruding side chains. We have compared several sets of compounds to assess the effect of possible steric hindrance by side chains. In the first series, we compared cefmetazole with its 7-α-H analog. There was a slight but significant retardation of penetration caused by the presence of the 7-α-methoxy group (Table 1). This cannot be due to the effect of hydrophobicity, because we found that the 7-α-H analog and cefmetazole were about equal in hydrophobicity (see Table 1). Thus, this effect is more likely to be caused by the protrusion of the methoxy group.

In the second series of experiments, we were intrigued by the observation that β-lactam compounds (such as ceftizoxime, cefotaxime, or cefuroxime) containing the substituted oxime side chain on the α-carbon of the substituent group at position 7 showed permeability much lower than

expected from their hydrophobicity values (Fig. 2). It is unlikely that the values of P_u were significantly erroneous, because at least one P_u value (for cefuroxime) was experimentally determined by us (see above). Again, the most likely possibility was the steric hindrance. In fact, it has been shown by the nuclear magnetic resonance technique that the side chain of this type forms a hydrogen-bonded ring structure with the amide hydrogen on C-7 and that this ring protrudes out of the main plane of the cephalosporin nucleus (10). The oxime substituent can exist as either the *syn-* (*Z*-) or the *anti-* (*E*-) isomer. Thus, we compared the biologically more active *syn-* compounds with *anti-* analogs which were not expected to produce this hydrogen-bonded ring structure. The *anti-* compounds penetrated several times more rapidly than did the *syn-* compounds, and the results suggest that the unexpectedly large effect of the substituent of this type is at least partially due to the formation of the rigid ring structure (Fig. 3). Interestingly, cefotaxime, whose calculated hydrophobicity is slightly lower than that of ceftizoxime, diffused more slowly than did the latter compound. Possibly, this is due to the larger size of cefotaxime, the effect of added size becoming very important in the compounds of this type that already have difficulty in fitting themselves into the pore.

It should be added that our results are quantitatively different from those of Kojo et al. (12), who concluded that *E. cloacae* outer membrane had a very high permeability toward ceftizoxime, about equal to the rate of permeation by cephaloridine. The discrepancy may be due to the use of different organisms. More significant, however, is the fact that their method, which uses a very involved inhibition procedure, could have produced results with significant errors. We noted that their data are internally inconsistent, and permeability coefficients that varied by more than an order of magnitude could be calculated for the same β-lactam, depending on which set of values are used.

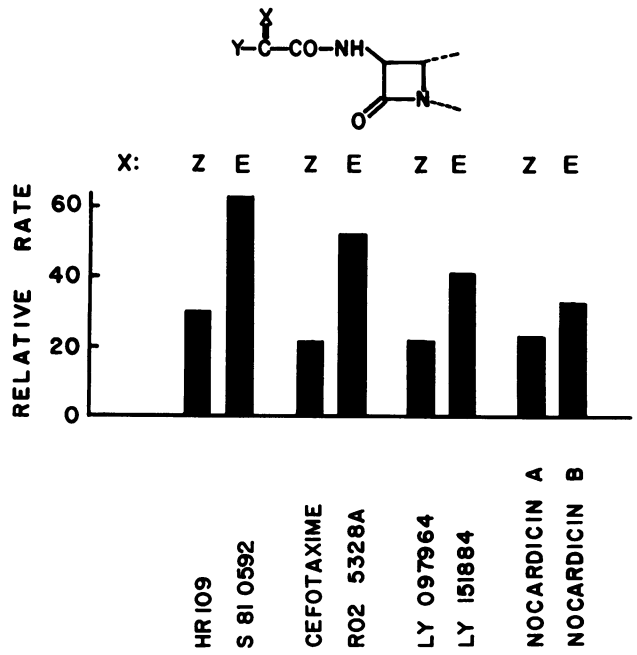

FIG. 3. Comparison of penetration rates of *syn-* (*Z*)- and *anti-* (*E*-) oxime-bearing compounds through the OmpF channel, determined by the liposome swelling assay.

In the third series of comparisons, we found that compounds with exceptionally bulky side chains, especially those with substituted ureido groups on the α-carbon of the position 7- side chain (such as cefoperazone, mezlocillin, and piperacillin) all had much lower permeability than expected from the hydrophobicity of the compounds (Fig. 2). Especially striking is the rather low permeability of cefoperazone, which is very hydrophilic ($P_u = -0.74$).

Zwitterionic compounds. As seen previously (20) with a few compounds, the presence of an additional cationic group resulted in a very strong acceleration of penetration through the "normal" *E. coli* porin channel (Fig. 4). Interestingly, the calculated hydrophobicity of the uncharged form of the compound did not seem to influence the permeability of the zwitterionic compounds much. Possibly, the presence of both positively and negatively charged groups made the overall surface of the compound hydrophilic enough.

Compounds with one positive and two negative charges. In general, compounds with one positive and two negative charges showed good permeability (Table 1), and again the penetration rate did not appear to show much dependence on the hydrophobicity of the uncharged form of the molecule (Fig. 4). This is probably because the properties of the surface of the molecule are dominated by the charged groups; as a result, the penetration rate can be much better than in the corresponding monoanionic compounds when the structure of the compound is predominantly hydrophobic (see SCE-796 in Fig. 4). The low permeability of ceftazidime was rather surprising; possibly, the negative charge on the substituted oxime structure has a particularly severe retardation effect due to its exposed position. In this connection, we noted that the permeability of azthreonam, which has the same substituted oxime structure, is also rather low despite its small monobactam nucleus (Table 1).

Compounds with two negative charges. Compounds with second anionic groups penetrated more slowly than did the monoanionic compounds of comparable hydrophobicity (Table 1). Among them, moxalactam had an exceptionally high permeability which was presumably due to the very hydrophilic nature of the rest of the molecule. Ceftriaxone also showed significant permeability despite its large size, possibly due to the very hydrophilic nature of its substituent at position 3.

Compounds with nontraditional nuclei. Two compounds tested had nuclei that belonged neither to the cephalosporin series nor to the penicillin series (Fig. 1). Imipenem had the highest permeability among the compounds tested, presumably owing to its compact structure and zwitterionic charge. This exceptionally high permeability may be of significance in the effectiveness of this drug against β-lactamase-producing *Enterobacter* strains, as described below. Azthreonam showed a rather high permeability among compounds carrying two negative charges, perhaps due to its compact monobactam nucleus. However, its penetration rate was only about one-tenth of that of imipenem, possibly due to the presence of the two negative charges and, more specifically, to the presence of a negative charge on the oxime substituent (see above).

Penetration through channels of other porins. We expect that the results obtained above by using mostly the OmpF porin of *E. coli* would be applicable at least qualitatively in the diffusion of β-lactams through the channels of other porins, because the interaction between the channel and the penetrating solute appears to be totally nonspecific. In fact, the general effect of the solute size, hydrophobicity, and charge was seen to be similar in our limited studies with

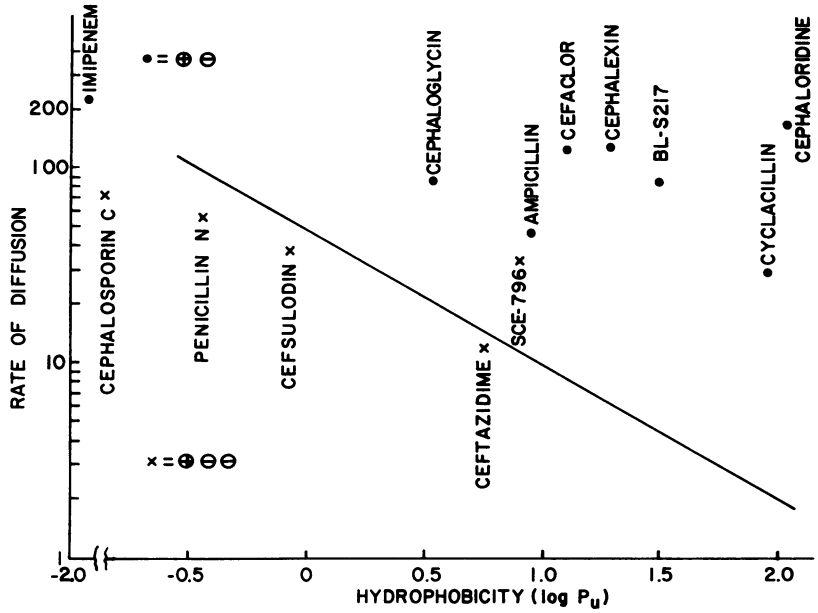

FIG. 4. Rates of penetration of zwitterionic compounds (●) and compounds with one positive and two negative charges (×). Penetration rates through the OmpF channel in the reconstituted liposomes are shown, with the rate of cephacetrile arbitrarily set as 100. The solid line shows the behavior of monoanionic cephalosporins (see the legend to Fig. 2 and reference 21).

liposomes containing *Brucella abortus* porin (data not shown) and the OmpC porin of *E. coli* (Table 1). There were clearly quantitative differences, however. With the OmpC channel, which is narrower than the OmpF channel (20), the bulky side chains and the double negative charges were much more restricting than those in the penetration through the wider OmpF channel (Table 1). This is potentially a significant point, because recent results suggest that *S. typhimurium*, for example, may produce only the narrower OmpC porin in the bodies of the host animal (18).

E. coli K-12 derepresses a "new" porin, PhoE, when starved for phosphate (29). This channel favors negatively charged compounds (for an example see references 20 and 21) and should show a different pattern of permeability from the set of β-lactams studied here. However, the properties of this channel do not appear to be relevant to chemotherapy, because serum contains ca. 1 mM inorganic phosphate (1), a concentration almost 10 times higher than the concentration needed to repress the production of this porin (29).

On the other hand, *Neisseria gonorrhoeae* porin is reported to produce an anion-selective channel (31). This channel is expected to show a different selectivity for the penetration of various β-lactams, and this property may indeed be related to the high sensitivity of this organism to a number of β-lactams, most of which are anionic molecules.

Diffusion of β-lactams into intact cells. As pointed out earlier (20, 21), the penetration rates of β-lactams in proteoliposomes generally mimic those into the intact cells. However, there is evidence that the presence of net negative charge(s) inhibits penetration into intact cells significantly more than it does the diffusion into liposomes. Thus, cephacetrile and cefsulodin, each with one net negative charge, diffused into OmpF-containing liposomes at rates corresponding to 60 and 22% of the rate of the zwitterionic cephaloridine (Table 1), whereas the influx rates into OmpF-containing intact cells were only 23 and 3%, respectively (21). Similarly, the dianionic SCE-20 diffused into proteoliposomes at 19% of the rate of influx of monoanionic cephacetrile in proteoliposomes (20), but the corresponding rate into intact cells was only 4% (21). This more severe retardation of the negatively charged compounds in intact cells may be related to the presence of the Donnan potential (interior negative) across the outer membrane (27); this potential will strongly inhibit the influx of any solute with a net negative charge.

Comparison between penicillins and cephalosporins. Penicillin nucleus is considerably more hydrophobic than the cephalosporin nucleus, and there is a difference of ca. 0.5 in the log P_u values between penicillin and its corresponding cephalosporin analog containing CH_2-O-CO-CH_3 substituent at position 3. Direct comparison between penicillins and cephalosporins was difficult in our assay system, because most of the penicillins commercially available were too hydrophobic for our systems and crossed the liposome membranes through the lipid bilayer regions (20). One comparison that can be made is between ampicillin and its cephalosporin analogs, cephaloglycin and cephalexin, which diffused through the porin channel at rates that were two to three times higher than those of ampicillin. Another pair is cephalosporin C and penicillin N, in which the penetration rate of cephalosporin C was ca. 30% higher. It seems likely that at least a part of this difference is due to the difference in the hydrophobicity of the nucleus.

It has been claimed that penicillins tended to penetrate through the outer membrane via a nonporin pathway (24), and the relatively high rate of penetration of ampicillin through the phospholipid bilayer (30) has been correlated with this notion. Since many penicillins have very poor permeability through the porin channel due to their high hydrophobicity and their size, it is conceivable that any leakage through a nonporin pathway would become a significant part of their penetration mechanism. This, however, does not necessarily indicate the presence of any specific mechanism for penicillin penetration. In fact, the high permeability of ampicillin through the phospholipid bilayer in comparison with cefazolin and cephaloridine (21) is quite

expected from the gross physicochemical properties of these compounds. Cefazolin cannot cross the bilayer without the simultaneous influx of the counter ion Na^+ which is known to have an exceedingly low permeability through lipid bilayers (2). Cephaloridine penetrates lipid bilayers poorly, presumably because its pyridinium moiety carries a permanent positive charge. In contrast, a significant portion of ampicillin can exist in the uncharged form that can cross the bilayer without much difficulty. At present, we see no reason for postulating "specific" mechanisms that distinguish cephalosporins from penicillins in their penetration through the outer membrane.

Relationship between the permeability and the efficacy of β-lactams. When we limit ourselves to the β-lactams of earlier generations, we see a very good correlation between the permeability and the efficacy of the drug against gram-negative organisms, especially the enteric bacteria. Thus, among the penicillin series, the zwitterionic charge structure introduced with ampicillin produced the first penicillin with reasonable penetration rates as well as efficacy. In the cephalosporin series, the zwitterionic cephaloridine had an exceptionally high penetration rate and also an excellent activity against gram-negative bacteria.

This correlation seems to hold at least partially in more recent compounds. Thus, the use of the aminothiazole side chain in ceftizoxime and cefotaxmime did indeed improve the permeability of cefuroxime and also produced compounds with exceptional activity against gram-negative bacteria. However, this correlation does not seem to be valid in a strict sense in many situations. For example, compounds with the substituted oxime side chains are not exceptionally fast in their penetration rate despite their extreme efficacy against gram-negative bacteria (Fig. 2). Even those compounds that showed low and unmeasurable permeability in our system (such as piperacillin) appear to have at least a reasonable activity toward enteric bacteria. Although the present results show only relative rates of permeation, our earlier study with intact cells gave us absolute values of permeability coefficients (21), and thus it becomes possible to calculate the rate of diffusion in terms of half-equilibrium time. As described earlier, the most permeable monoanionic β-lactam (such as cephacetrile) has a half-equilibration time of 0.1 s. Since the lowest rate of diffusion we could measure was ca. 5% of this rate, the half-equilibration time would be ca. 2 s. Even if we assume that some of the compounds with "immeasurably low" penetration rates had rates one order of magnitude lower than this, the half-equilibration time woud still be less than 30 s, a far shorter time interval than the generation time of the organism. Clearly then, the high rates of penetration were important for the first generation agents because they were very rapidly hydrolyzed by the periplasmic β-lactamases. If the compound is very stable toward most β-lactamases as are some of the third generation agents, even a very slow penetration through the outer membrane would suffice to build up an effective periplasmic concentration.

Is there any benefit that can be derived from designing β-lactams of high permeability? Indeed, there seem to be some important benefits. First, there are recent reports of bacterial mutants that are resistant to β-lactams due to their reduced outer membrane permeability, often caused by alterations in the production of porins (9, 11, 13; Medeiros and Nikaido, quoted in reference 18) or what appear to be porins (7). In this situation, obviously the agents with intrinsically high rates of penetration would have an advantage. In mutants of *E. coli* produced in the laboratory, the mutants became resistant only to those agents with lower rates of penetration due to the presence of a double negative charge or high degree of hydrophobicity (9); the strains remained fully sensitive to agents such as cephaloridine with a high rate of diffusion through the pore. Secondly, it has been discovered recently that *E. cloacae* strains constitutively producing chromosomally determined β-lactamase are resistant to most third generation β-lactams despite the inability of the enzyme to hydrolyze these agents (23, 25, 28). This was proposed to be due to the "trapping" of the β-lactam molecules by the β-lactamase enzyme molecules without hydrolysis (28). This idea was challenged on the ground that a number of β-lactamase molecules larger than the number of β-lactams per milliliter of culture must be present for this mechanism to work (25). However, this argument disregards the presence of an outer membrane barrier. If we assume the permeability coefficient to be ca. 10^{-6} cm/s, i.e., 1% (Table 1), and the external concentration to be 2 μg/ml, i.e., 0.4×10^{-8} mol/ml (with the β-lactam molecular weight assumed to be 500), the maximal rate of diffusion of this β-lactam through the outer membrane V is $V = P \times A \times C_0$; P, A, and C_0 denote permeability coefficient, area of the membrane, and external concentration of the diffusing solute, respectively. With a value of 132 cm^2/mg for A (14), $V = 10^{-6}$ (cm/s) $\times$ 132 (cm^2/mg) $\times$ 0.4×10^{-8} (mol/cm^3) $\times$ 6.023×10^{23} (molecules per mol)/(4×10^9 cells per mg) = 80 molecules per cell per s if we assume that 1 mg (dry weight) corresponds to 4×10^9 cells. Some major periplasmic proteins, such as the maltose-binding protein, exist in copies of 4×10^5 copies per cell (4). If we assume that the number of β-lactamase molecules is comparable, and if we assume the generation time to be 20 min, the cell should be making 333 molecules per s ($4 \times 10^5/20/60 = 333$) which would be more than enough to bind all of the incoming β-lactam molecule every second. If the porin of *Enterobacter* strains is less permeable than that of *E. coli*, the trapping would work even more efficiently. Thus, the trapping is a theoretically possible mechanism. On the other hand, if the β-lactam is a rapidly penetrating compound with a high permeability coefficient, this mechanism cannot catch up with the rate of influx of the agent. It is interesting that imipenem, which has an exceptionally high permeability coefficient among the newer agents, remains very effective against the β-lactamase-producing *Enterobacter* strains (25). These calculations, however, do not show that the resistance is indeed caused by the trapping mechanism, and experimental studies are needed to exclude alternative mechanisms. However, even if the resistance is due to the slow hydrolysis of the third generation agents, it is clear that such slow rates of degradation would not be able to lower the periplasmic concentration of the drug effectively without the very slow rates of influx through the outer membrane, and these observations again emphasize the importance of creating drugs with intrinsically high penetration rates through the outer membrane.

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