PHOSPHOLIPID BILAYER PERMEABILITY OF β-LACTAM ANTIBIOTICS

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(Received for publication September 4, 1982)

Liposomes containing penicillinase or cephalosporinase were prepared from the phospholipids of *Escherichia coli*. After free β-lactamase was inactivated by clavulanic acid or penicillanic acid sulfone followed by separation of inactivated enzyme and inhibitor from liposomes by gel filtration, the permeability of these liposomes to ampicillin, cefazolin and cephaloridine was estimated by measuring the hydrolysis of these antibiotics by the entrapped enzymes. The permeability parameter C (minute^{-1} μM lipid^{-1}) of ampicillin, cefazolin and cephaloridine was calculated to be 2.35×10^{-4}, 0.33×10^{-4} and 0.52×10^{-4}, respectively.

The lipid bilayer permeability of these antibiotics was also measured by using the liposomes containing these antibiotics. About half of the initially entrapped ampicillin was released from the liposomes within 80 minutes, while no significant release of cefazolin and cephaloridine could be detected during the same period.

These results clearly indicates that the lipid bilayer membrane is more permeable to ampicillin than cefazolin and cephaloridine, and they are consistent with the observations of SAWAI *et al.*[1], who showed that ampicillin was a more effective antibacterial drug than cefazolin and cephaloridine against the porin-deficient mutants.

The ability of β-lactam antibiotics to pass through the bacterial outer membrane is an important factor for their efficacy as antibacterial drugs.

The permeability of β-lactam antibiotics to intact cells was first measured by SAWAI *et al.*[2] and ZIMMERMAN & ROSSELET[3] by using the enzyme activity of β-lactamase located in the periplasm. In the wild type strains, antibiotic permeability depends on the hydrophilicity of the molecules[4,5]; for the cephalosporins, the permeability depends also on the charges of the compounds[5]. NIKAIDO *et al.*[6] and ALPHEN *et al.*[7] showed that cephaloridine uses the porin pores as permeation route in *Salmonella typhimurium* and *Escherichia coli*. We also suggested that cephalosporins pass through the outer membrane *via* the porin pore even in *Proteus mirabilis* and *Enterobacter cloacae*[1]. However, we hypothesized in our previous paper[1] that a pathway other than the porin pore might play a significant role in outer membrane permeation of penicillins, since the decrease in susceptibility of porin-deficient mutants to the penicillins was significantly smaller than with cephalosporins. In fact, significant amounts of penicillins could penetrate the porin-deficient mutants[1,7]. A possible pathway for penicillins is through the lipid bilayer of the outer membrane, but it is not easy to show that a penicillin can penetrate the lipid bilayer of intact cells because the outer membrane contains various materials that could interfere with the assay.

Phospholipids extracted from *E. coli* can form closed vesicles such as liposomes without lipopolysaccharides[8]. The liposomes may be a useful model of the bacterial hydrophobic barrier composed of a lipid bilayer. When β-lactamase is entrapped in the liposome and concealed from the substrates outside the liposome, it is possible to estimate the lipid bilayer permeability by measuring the enzyme activity. We succeeded in preparing liposome-entrapped penicillinase and cephalosporinase. Subsequently, we succeeded in enclosing the antibiotics themselves in the liposomes. In this paper, we show that

these liposomes can serve as a model system to examine the penetration of ampicillin, cefazolin and cephaloridine through the lipid bilayer membrane.

Materials and Methods

Bacterial Strains

Escherichia coli CS109 which is an *E. coli* K12 substrain was a gift from Dr. A. P. PUGSLEY[9]. *Citrobacter freundii* GN346 is a strain which produces its species specific cephalosporinase semiconstitutively[10]. *E. coli* ML1410/RGN823 is a strain harboring an R plasmid RGN823 which mediates the constitutive synthesis of type Ib penicillinase[11] in the host bacteria.

β-Lactam Antibiotics and Their Related Compounds

Penicillins and cephalosporins were kindly provided by the following pharmaceutical companies: ampicillin and 6-aminopenicillanic acid, Meiji Seika Co., Tokyo, Japan; cefazolin, Fujisawa Pharmaceutical Co., Osaka, Japan; cephaloridine, Torii Phamaceutical Co., Tokyo, Japan. Clavulanic acid was a generous gift of Beecham Pharmaceuticals, Blockham Park, Betchworth, Surrey, U.K.. Penicillanic acid sulfone (CP45,899) was synthesized from 6-amino-penicillanic acid in our laboratory.

Preparation of Phospholipids and β-Lactamases

Bacterial phospholipids were extracted from *E. coli* CS109 by the method of BLIGH & DYER[12] and purified by subsequent chromatography on silica gel. Cardiolipin was obtained from Sigma Co. Lipid concentration was determined by measuring the amount of its fatty acid ester according to RAPPORT & ALONZO[13].

Penicillinase and cephalosporinase were prepared from *E. coli* ML1410/RGN823 and *C. freundii* GN346, respectively. These β-lactamases were completely purified by absorption and elution on a Sephadex ion-exchange column and by gel filtration on a Sephadex G-75 column; purity was confirmed by SDS polyacrylamide slab gel electrophoresis.

Preparation of the Liposomes with Entrapped β-Lactamase or β-Lactam Antibiotic

A mixture of 4 mg of *E. coli* phospholipids and 0.37 mg of cardiolipin in a test tube was dried from chloroform solution to a thin film using nitrogen gas under vacuum for 2 hours. To the test tube, glass beads (1 mm diameter) and 0.4 ml of phosphate buffered saline (PBS, pH 7.0) containing 10 nmoles penicillinase were added. The PBS composed of 1.47 g dipotassium phosphate, 0.7 g monopotassium phosphate and 6.8 g sodium chloride per liter. The preparation was then mixed vigorously using a Vortex mixer at room temperature and finally briefly sonicated with a Branson water bath sonicator. The liposome preparation was incubated with 5 μmoles of clavulanic acid (500 molar excess with respect to the enzyme) at 30°C for 60 minutes in order to inactivate the untrapped enzymes. (The merits of this inactivation procedure will be discussed below.) The residual enzymes and inhibitors were separated from the liposomes by gel filtration on a Sepharose CL-6B (1.0×55 cm) equilibrated with PBS.

Cephalosporinase-enclosed liposomes were prepared by the same procedure, except that 2.8 nmoles cephalosporinase and 560 nmoles of CP45,899 were used instead of penicillinase and clavulanic acid.

Liposomes containing β-lactam antibiotics were made as follows. Two milligrams of *E. coli* phospholipids and 0.14 mg of cardiolipin were dried in a test tube as described above. A PBS solution (0.2 ml) containing 10 mM glucose and 20 mM antibiotic was added to the dried lipids in the tube; then the preparation was mixed using a Vortex mixer and sonicated as mentioned above. Untrapped antibiotic was removed from the liposomes by gel filtration on a Sephadex G-25 column.

Uptake of β-Lactam Antibiotics into Liposomes

The mixture of 0.6 ml of 0.5 mM antibiotic solution and 5.1 ml of PBS was preincubated at 30°C for 5 minutes. Then 0.3 ml of liposome suspension ($OD_{280}=1.0$) was added to the mixture and the incubation was continued at the same temperature. Starting from the time of addition of the liposomes, 1 ml samples were withdrawn at different time intervals and the amount of hydrolyzed antibiotic was measured by a micro-iodometric method[14] with modification. In this method, hydrolyzed product remaining inside the liposomes could also be detectable, because the iodine consumption remained constant before

and after complete disruption of the liposomes by sonication.

Two control experiments were performed at the same time. One of them was the enzymic hydrolysis of β-lactam antibiotics by the liposomes disrupted in the presence of 0.05% Triton X-100. It was also confirmed that the enzyme was not affected in its activity by Triton X-100. Another control was the hydrolysis of β-lactam antibiotics by the free enzymes outside the liposomes. After the liposome suspension was centrifuged at 100,000×*g* for 30 minutes, the enzyme activity in the supernatant was measured. Net rate of hydrolysis of β-lactams by the entrapped enzymes was calculated by the subtraction of the hydrolysis by the supernatant from that by the liposome suspension.

Efflux of β-Lactam Antibiotics from Liposomes

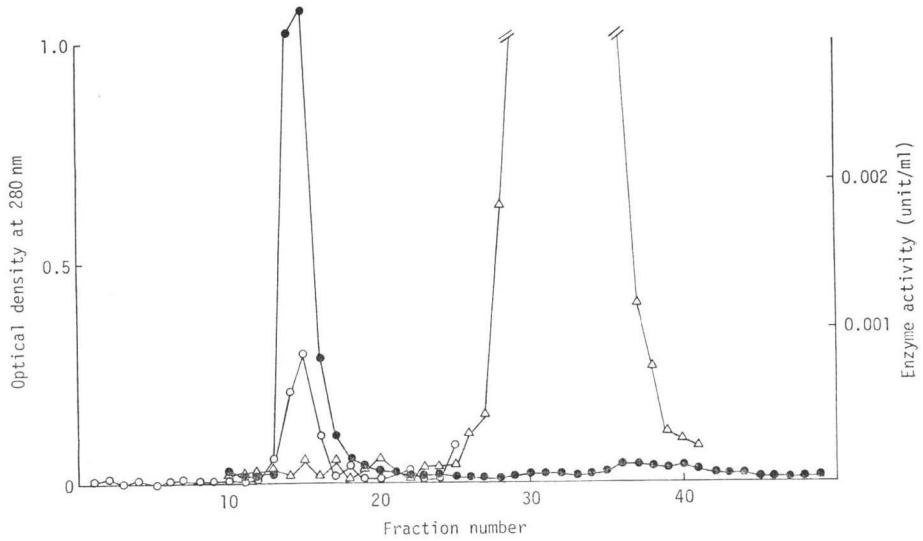
Antibiotic-enclosed liposomes (OD_{280}=1.0) were used immediately after being fractionated on the Sephadex G-25 column. The liposome fraction was diluted ten times with PBS and incubated at 30°C. At different time intervals, 0.4 ml of incubation mixture was withdrawn and antibiotic that had leaked out was assayed by micro-iodometry after complete hydrolysis of the antibiotic by short incubation with excess penicillinase or cephalosporinase at 30°C for 5 minutes. Total antibiotic in the reaction mixture was determined after disruption of the liposomes by 0.05% Triton X-100, by the same way as described above. Entrapped antibiotic was calculated by subtraction of leaked antibiotic from total antibiotic.

Results

Preparation of β-Lactamase-containing Liposomes from the Phospholipids of *Escherichia coli*

Fig. 1 shows a typical profile of penicillinase-containing liposomes prepared as described in Materials and Methods but in the absence of clavulanic acid. Enzyme activity was assayed using cephaloridine as substrate, because it is believed to have low permeability for the lipid bilayer from the observations in intact cells[1]. Liposomes were detected by light scattering at 280 nm. They were clearly separated from free enzyme by the gel filtration. In so far as measuring the enzyme activities with cephaloridine, the liposome fractions showed very low activity. However, when the liposomes were disrupted by addi-

Fig. 1. Elution profile of penicillinase-containing liposomes on a Sepharose CL-6B column (1.0×55 cm). The liposomes were prepared as described in Materials and Methods except that clavulanic acid was not added. Fractions of 0.5 ml were collected. Enzyme activity was measured by using 200 μM cephaloridine as a substrate in the absence (△) or the presence (○) of 0.1% Triton X-100. Closed circles represent the optical density at 280 nm.



tion of 0.1% Triton X-100, the enzyme activity showed about a 6-fold increase. This fact indicates that most the penicillinase entrapped in the liposomes was hidden inside the lipid bilayers. Liposome fraction contained 0.5% of the enzyme activity initially added as the cryptic enzyme. We were also able to obtain liposomes which entrapped cephalosporinase; in this case, the liposome fraction held 0.6% of the enzyme activity initially added.

When the penicillinase-containing liposomes were used for assay of ampicillin uptake at low antibiotic concentration (50 μM), intact liposomes showed almost the same enzyme activity as that of the Triton X-100 treated liposomes. We noticed that a slight amount of β-lactamase which had escaped separation on the gel column significantly interfered with the measurement of the uptake of β-lactams by the liposomes. Therefore, the untrapped penicillinase was irreversibly inactivated by clavulanic acid prior to the gel filtration. Clavulanic acid is an effective suicide inactivater for the type Ib penicillinase[15]. It decreased the free penicillinase activity outside the liposomes to 4.5% of its original activity when a 500 molar excess of inhibitor to the enzyme was used at 30°C for 60 minutes. Although clavulanic acid partially inactivated the enzymes inside the liposomes, the degree of inactivation was lower than the inactivation to the free enzymes probably due to the permeability barrier of the liposomal membrane to clavulanic acid. After the clavulanic acid treatment, 23% of the entrapped enzyme was still active. Therefore, the interference of free enzyme with the assay for the permeability of β-lactams could be diminished by the clavulanic acid treatment.

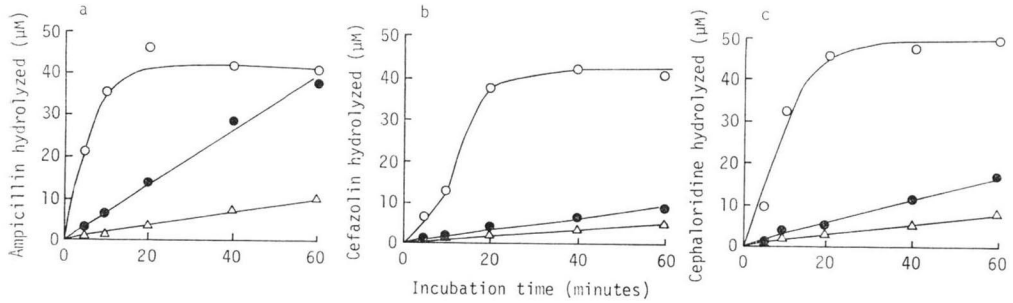
In the case of cephalosporinase, penicillanic acid sulfone (CP45,899)[16] was used as an inhibitor because we found that this compound was a much more effective inhibitor of the cephalosporinase[17]. Two hundred molar excess of CP45,899 to the enzyme showed a similar effect on the enzyme activity as in the case of penicillinase described above and 27% of the entrapped enzyme activity was still active after treatment.

Uptake of Ampicillin, Cefazolin and Cephaloridine into the Liposomes

The time course of ampicillin hydrolysis by liposome-entrapped penicillinase was measured (Fig. 2a). The initial rate of hydrolysis by the supernatant of the liposome suspension (0.13 μM/minute) was only 3% of that by the disrupted liposomes (4.22 μM/minute) when the initial concentration of ampicil-

Fig. 2. Uptake of ampicillin, cefazolin and cephaloridine into liposomes.

The preparation of enzyme-containing liposomes and the assay of antibiotic uptake were as described in Materials and Methods. (a) Ampicillin hydrolysis by penicillinase-containing liposomes. (b) Cefazolin and (c) cephaloridine hydrolysis by cephalosporinase-containing liposomes. Phospholipid concentrations in the assay mixture were 47 μM (a) and 50 μM (b and c). Symbols: ○, hydrolysis in the presence of 0.05% Triton X-100; ●, hydrolysis in the absence of Triton X-100; △, hydrolysis by the supernatant obtained by centrifugation of the liposome fraction at 100,000×g for 30 minutes.



lin was 50 μM and the concentration of the liposome was 47 μM. On the other hand, intact liposomes showed relatively high activity and hydrolyzed ampicillin at the rate of 0.65 μM/minute in the same condition. The difference between the rate of hydrolysis by intact liposome and the supernatant represents the rate of hydrolysis by the entrapped enzyme inside the liposome.

The antibiotic concentration inside the liposomes could be estimated according to the equation of SAWAI *et al.*[2] using the same postulations, as follows:

$$Si=\frac{v_i}{v_d}\left(\frac{Km\cdot So}{Km+So-So\cdot(v_i/v_d)}\right) \quad \text{(i)}$$

where, v_i and v_d are the net rate of hydrolysis of antibiotics by intact liposomes and disrupted liposomes, respectively, obtained by the subtraction of the rate of hydrolysis by the supernatant. Si and So represent the antibiotic concentration inside and outside the liposomes, respectively, at the steady state during the assay.

Permeability parameter C could also be calculated according to the equation of ZIMMERMAN & ROSSELET[3] by using the value of Si obtained above.

$$C=\frac{1}{So-Si}\cdot\frac{Vmax\cdot Si}{Km+Si}/[\text{phospholipid concentration}] \quad \text{(ii)}$$

Permeability parameter C was normalized by the phospholipid concentration in the assay mixture instead of normalization by the surface area of the liposome because it was difficult to determine the accurate value of the area and the surface area should be proportional to the phospholipid concentration in so far as the conditions of the preparation of liposomes were kept constant. Although *Vmax* could be determined experimentally by measuring the enzyme activity of the disrupted liposomes in the presence of excess substrate, we calculated *Vmax* using the following modified Michaelis equation.

$$Vmax=\left(1+\frac{Km}{So}\right)\cdot v_d \quad \text{(iii)}$$

In the presence of 50 μM ampicillin outside the liposomes, Si was 2.73 μM and the permeability parameter was calculated to be 2.35×10^{-4} minute^{-1} μM lipid^{-1} (Table 1). Si value depended on the concentration of the antibiotic outside the liposomes, whereas the permeability parameter was independent to the antibiotic concentration outside the liposomes (data not shown) ranging from 25 μM to 200 μM. This result confirms that the parameter calculated above corresponds to the diffusion coefficient of ampicillin to the lipid bilayer membrane. For example, if there were any contribution of the enzyme attached to the outside surface of the liposome, the permeability parameter calculated by equation (ii) should depend on the antibiotic concentration outside the liposomes. It was not the case.

The assay of the uptake of cefazolin and cephaloridine into the penicillinase-containing liposomes was difficult because of the low affinity of the penicillinase for these cephalosporins.[18] Therefore, we employed the cephalosporinase-containing liposomes for measuring the uptake of cephalosporins into liposomes; free cephalosporinase was inhibited by CP45,899 instead of clavulanic acid as described above.

Table 1. Antibiotic concentrations inside the liposomes and permeability parameters.

Antibiotic	Lipid conc. (μM)	v_i/v_d	*Km* (μM)	*Vmax* (μM/minute)	Si (μM)	Permeability parameter C (minute^{-1} μM lipid^{-1})
Ampicillin	47	0.217	33	6.82	2.73	2.35×10^{-4}
Cefazolin	50	0.046	169	7.60	1.79	0.33×10^{-4}
Cephaloridine	50	0.040	14	4.15	0.45	0.52×10^{-4}

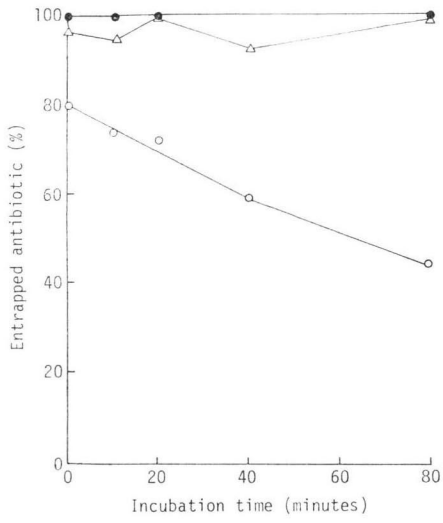
Fig. 2b and 2c show the time course of hydrolysis of cefazolin and cephaloridine, which were measured in a similar manner as that described above. The permeability parameters of cefazolin and cephaloridine were calculated to be 0.33×10^{-4} and 0.52×10^{-4} minute^{-1} μM lipid^{-1}, respectively. These values were significantly lower than that for ampicillin (Table 1). These permeability parameters should be independent to the nature of the enzymes in so far as the physico-chemical difference of the enzymes did not affect the characteristics of the liposomes. In fact, we preliminally obtained almost the same permeability parameter for cephalothin to either penicillinase- or cephalosporinase-containing liposomes (0.1×10^{-4} minute^{-1} μM lipid^{-1}). (It was possible to measure cephalothin hydrolysis by either the penicillinase or the cephalosporinase.) Therefore, there may be no great difference of physico-chemical characteristics between penicillinase- and cephalosporinase-containing liposomes.

Efflux of Ampicillin, Cefazolin and Cephaloridine from Liposomes

In order to confirm the observed marked difference in lipid bilayer permeability between ampicillin and cephalosporins, we prepared liposomes containing ampicillin, cefazolin and cephaloridine. After gel filtration, the amount of antibiotics inside and outside the liposomes was determined as described in Materials and Methods. The ampicillin-liposome fraction was found to contain 44 nmoles ampicillin/ml in the interior of the liposomes and 11 nmoles ampicillin/ml outside the liposomes immediately after gel filtration. On the other hand, cefazolin liposomes and cephaloridine liposomes were found to contain 33 nmoles cefazolin/ml and 50 nmoles cephaloridine/ml inside the liposomes, respectively, whereas free cephalosporins could hardly be detected in both cases. The presence of free ampicillin in the liposome fraction may indicate the release of ampicillin from liposomes during gel filtration.

Fig. 3. Release of ampicillin, cefazolin and cephaloridine from liposomes.

Entrapped antibiotic was calculated by subtraction of the leaked antibiotic concentration, which was measured as described in Materials and Methods, from the total antibiotic concentration determined enzymatically after disruption of the liposomes by addition of 0.05% Triton X-100. It is expressed as a relative amount against the total amount of antibiotic in the reaction mixture. Symbols: ○, ampicillin; ●, cefazolin; △, cephaloridine.



The three types of liposomes were shown to contain about the same amount of glucose, which was initially added as an index of the internal volumes of the liposomes; its amount was determined according to KINSKY *et al.*[10]. Therefore, the difference in the amount of the trapped antibiotics could not be attributed to a difference in the internal volume of the liposomes. The difference may be attributed to ionic interaction between the antibiotic and the lipid bilayer, since the decreasing order of trapping was cephaloridine and ampicillin>cefazolin; the latter has a negative charge similar to the surface of the lipid bilayer.

When the ampicillin-containing liposome fraction was diluted 10-fold with PBS and incubated at 30°C, the entrapped ampicillin was gradually released from the liposomes (Fig. 3). This release followed pseudo-first order reaction. However, the amount of cefazolin and cephaloridine released represented less than 6% of trapped antibiotics even after 80 minutes of incubation.

This result strongly confirms the higher permeability of ampicillin than that of cephalosporins to lipid bilayer membrane.

Discussion

β-Lactamase-containing liposomes were prepared from *E. coli* phospholipids, and it was demonstrated that these liposomes could be used to assay the penetration of β-lactam antibiotics through a lipid bilayer membrane. The principle of the method used for measuring the antibiotic uptake into liposomes is essentially the same as the method of SAWAI *et al.*[2] and ZIMMERMAN & ROSSELET[3]. These two methods were originally devised for measuring penetration of antibiotics through the bacterial outer membrane making use of the β-lactamases located in the periplasm. In this study, β-lactamases were localized inside the liposomal membrane. If untrapped enzyme activity is completely removed, the rate of antibiotic hydrolysis by the liposomes should depend on the penetration of the antibiotics through the lipid bilayer until the antibiotic concentration in the liposome is saturated with respect to the enzyme reaction.

In practice, we encountered some difficulty, namely, free enzymes remaining in the liposome fractions interfered with the assay. However, this problem could be overcome by inactivation of the free enzymes with β-lactamase inhibitors prior to gel filtration. When more than 95% of the free enzyme was irreversively inactivated by the inhibitor treatment, about three quarters of the trapped enzymes also lost its activity because the inhibitor penetrated the liposomes. However, the remaining enzyme activity in the liposomes was sufficient to assay the penetration of the antibiotics through the lipid bilayer.

The results of this study demonstrated that ampicillin could penetrate the lipid bilayer membrane four or seven times faster than cephaloridine and cefazolin. When the cephalosporins were compared, cephaloridine showed higher permeability than cefazolin. Although the difference in permeability was clear among the three antibiotics, there was a possibility that observed difference might be due to the difference of the physico-chemical effect of the two β-lactamases in the lipid bilayer. This possibility could be eliminated by two reasons. One is the experiment of cephalothin uptake into penicillinase- and cephalosporinase-liposomes as described in Results. The other is the efflux experiment. In the latter case, the nature of the enzymes was independent on the properties of the liposomes. The results from these experiments supported the conclusion that ampicillin has higher permeability for the lipid bilayer than cefazolin and cephaloridine.

These findings are of interest in connection with the observations reported previously[1]. When an *E. coli* strain lost porin, MIC values of cephaloridine and cefazolin increased 8 and 16 times, respectively. However, the MIC value of ampicillin against the mutant was only two times that of the wild strain. The changes in susceptibility to ampicillin and the cephalosporins were compatible with alteration in the outer membrane permeability of ampicillin and the cephalosporins in the mutant. These results strongly suggested that there was an additional route for ampicillin permeation of the outer membrane other than porin pore.

When the β-lactam antibiotics penetrate the outer membrane *via* a pathway other than porins, they may encounter two different kinds of barriers[20]. One of them is a hydrophilic barrier mainly composed of polysaccharide chain region. It can be presumed that many hydrophobic compounds are effectively rejected by this layer. Ampicillin is one of the hydrophilic antibiotics and its hydrophobicity is almost the same as these two cephalosporins[1]. Therefore, it may be able to permeate this layer. The second barrier is a hydrocarbon chain region. Our findings in this study suggest that ampicillin appreciably permeates this layer, whereas cephalosporins are rejected.

These findings suggest that unknown factors such as electron distribution or steric properties of β-lactam antibiotics may play an important role for lipid bilayer permeation of these compounds. As a result, their permeability may not be apparently proportional to their hydrophobicity.

It should also be emphasized that the liposome system, with some compositional modifications, may be used as a simple tool for the *in vitro* analysis of the mechanism of oral β-lactam-antibiotic penetration through the outer layer of intestinal tract.

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