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The use of liposomes as a model for drug absorption: β-lactam antibiotics

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Liposomes were prepared from egg phosphatidylcholine–cholesterol–diacetylphosphate (80 : 20 : 5) and total lipid extracts of rat intestinal mucosa, and the permeability of the liposomal membrane to eight β-lactam antibiotics was studied by using a dynamic dialysis method. Although all the antibiotics used here are ionized and poorly lipid-soluble at pH 6·5, some of them are orally active and efficiently absorbed from the small intestine. The release rate constants from the aqueous dispersion of drug-entrapped liposomes were approximately in the order of their absorbability. Intestinal lipid liposomes were more permeable to the antibiotics than egg lecithin liposomes and the release rate constants for the drugs from intestinal lipid liposomes were strongly correlative with their absorption rate constants, except for cephalothin and ampicillin, the deviations of which could be explained by their surface activity. It is suggested that lipid components of the intestinal mucosa and the bilayer structure may play an important role in the absorption process of the antibiotics. The validity of liposomes as a model for the intestinal absorption of drugs is also discussed.

β-Lactam antibiotics given orally are efficiently absorbed (Cole et al 1972; Hertz 1973), though they are ionized at the pH of the small intestine. Although basic studies on the intestinal absorption of β-lactam antibiotics have been made (Penzotti & Poole 1974; Yasuhara et al 1977; De Young et al 1978; Tsuji et al 1978), the mechanism by which the ionized forms are absorbed remained unresolved.

We have shown that the rapid absorption of cephalexin, which is hydrophilic, can be explained by its high transfer rate across lipid bilayers (liposomes) by using cefazolin as a reference (Yasuhara et al 1977). The present study deals with the permeation of some β-lactam antibiotics across liposomal membranes. Permeation rates are compared with the in situ absorption rates from rat small intestine to obtain further information on the transfer mechanism of β-lactam antibiotics across the intestinal boundary. The validity of liposomal membranes in early screening of new drugs is also discussed.

MATERIALS AND METHODS

Materials

Ampicillin anhydrous (Takeda Chemical Industries, Osaka), amoxicillin anhydrous, cephalexin anhydrous, sodium cefazolin anhydrous, sodium ceftezol anhydrous (Fujisawa Pharmaceutical Co., Osaka), sodium cephalothin anhydrous (Shionogi Pharmaceutical Co., Osaka), cephaloridine anhydrous (Torii Chemical Co., Tokyo), cephradine anhydrous (Sankyo Co., Tokyo) were used as supplied. Egg phosphatidylcholine was prepared from egg yolks according to the method of Rhodes & Lea (1957). Total lipids were extracted from the scraped mucosa of rat small intestine by the method of Folch et al (1957). Other chemicals were of the highest grade available.

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Preparation of drug solution

For liposomal and absorption studies, drugs were dissolved in isotonic phosphate buffered saline (Na_2HPO_4-NaH_2PO_4-NaCl, pH 6·5) and the isotonic phosphate buffer (Na_2HPO_4-NaH_2PO_4, pH 6·5), respectively.

Apparent partition coefficient

Chloroform-phosphate buffer (pH 6·5) partition coefficients were determined according to Kakemi et al (1967a).

Drug partitioning to chloroform containing lipids

Drug partitioning between water and chloroform containing total lipid extracts of rat intestinal mucosa was determined according to Furusawa et al (1972).

Preparation of liposomes

Two kinds of liposomes were prepared, one from egg phosphatidylcholine (80), cholesterol (20), and dicetylphosphate (5 μmol), and the other from total lipid extracts of the intestinal mucosa (32 mg) according to Kinsky et al (1968). Briefly, the lipid

mixture or the total lipid extracts dissolved in chloroform were pipetted into 25 ml round-bottomed flasks. The chloroform was removed under vacuum using a rotary evaporator. To the thin, dry lipid film, 5 ml of drug solution (drug concentration, 20 mM) was added. Mechanical shaking with a Vortex mixer for 10 min caused complete dispersion of the lipids. The suspension was then sonicated (Ohtake Sonicator-150, Japan) for 2·5 min on ice. A Sephadex G-25 column was used to separate the liposomal fraction from free drugs and the liposomal suspension was immediately used for transfer experiments.

Transfer rate experiments

The overall transfer rate of drugs from liposomal suspensions was determined by the modified method of dynamic dialysis of Meyer & Guttman (1970). Six ml of liposomal suspension containing a drug was added into the Visking cellulose tube (20/32) and external solution was 60 ml of isotonic phosphate buffered saline. Temperature was maintained at 37 °C.

Egg phosphatidylcholine and intestinal total lipid extracts used in all these transfer experiments were each from a single preparation to avoid any variation in the liposome.

Absorption experiments

Male Wistar albino rats 160–200 g were used as described by Kakemi et al (1967b). The animals had their bile duct ligated and were maintained at 37 °C. Forty ml of drug solution (0·1 mM, pH 6·5) was recirculated in the small intestine at the rate of 5 ml min^{-1}. After 1 h, the solution was removed and the intestine washed with pH 6·5 phosphate buffer. The washings were combined with the perfusate and made up to 100 ml with buffer. The amount of drug absorbed was calculated by difference.

For amoxicillin, the effect of pretreatment with $HgCl_2$ was also examined as described by Kimura et al (1978). The small intestine was pretreated with 3 mM $HgCl_2$ for 5 min before the absorption experiment to modify the apical plasma membrane of the epithelial cells.

The results were expressed as the mean with standard deviation of at least four experiments.

Analytical methods

In transfer experiments, ampicillin and amoxicillin were determined spectrofluorometrically by the method of Miyazaki et al (1974, 1977, respectively), and cephalothin, cephaloridine, cephradine, cephalexin, ceftezol, and cefazolin were determined spectrophotometrically at 236, 240, 262, 262, 271, and 271 nm, respectively. In absorption experiments, a high pressure liquid chromatograph TRI ROTAR (Japan Spectroscopic Co.) was used in a reverse phase with a SC-02 (ODS type) (Japan Spectroscopic Co.). Mobile phases of methanol in water containing 0·01 M ammonium acetate were used, and flow rates were maintained at 1 ml min^{-1}. The two penicillins were detected at 214 nm, and the cephalosporins at corresponding wavelengths. Preparation of samples for h.p.l.c. was as reported by Kimura et al (1978). The drug concentration was calculated from the peak height using the calibration curve. Glucose was determined by Glucose·HK·Test (Glucoquant, Boehringer Mannheim Yamanouchi Co.).

Surface tension measurements

Surface tension measurements were made at 25 °C using a Du Noüy Tensiometer (Shimadzu, Japan).

RESULTS AND DISCUSSION

Intestinal absorption of β-lactam antibiotics

The results of the absorption experiments are summarized in Table 1. Ampicillin, amoxicillin, cephalexin, and cephradine are orally active, while the other antibiotics are used parenterally. All would be virtually completely ionized at the pH of the small intestine, cephalexin, cephradine, cephaloridine, and aminopenicillins being mostly zwitterions, the others anions. Amoxicillin has been shown to be absorbed by concentration-dependent, carrier-

Table 1. Absorption of β-lactam antibiotics from rat small intestine in situ. 40 ml of drug solution (0·1 mM in pH 6·5 isotonic sodium phosphate buffer) was perfused in the small intestine of the anaesthetized rat for 1 h, and the absorption was calculated from the reduction of the drug in the perfusion solution. Results are expressed as the mean (with s.d.) of 4–5 experiments.

Drug	P.C.*	% absorbed in 1 h
Ampicillin	0·018	9·7 (1·1)
Amoxicillin	0·004	15·4 (2·2)
Amoxicillin, $HgCl_2$-treated**		6·8 (2·3)
Cephalothin	0·039	15·0 (2·4)
Cephaloridine	0·033	7·1 (1·4)
Cephradine	0·008	33·8 (4·2)
Cephalexin	0·006	23·2 (2·3)
Ceftezol	0·030	10·3 (1·4)
Cefazolin	0·006	5·0 (1·1)

* $CHCl_3$-to-phosphate buffer (pH 6·5) partition coefficients.

** Small intestine was pretreated with 3 mM $HgCl_2$ for 5 min.

mediated transport systems from rat small intestine (Tsuji et al 1977; Kimura et al 1978). Pretreatment of the intestinal mucosa with 3 mM $HgCl_2$ for 5 min reduced the absorption (Kimura et al 1978) and it seemed that the irreversible modification of the mucosal SH groups blocked the carrier systems, thus the remaining portion of the absorption would be by simple diffusion. The absorption of the other antibiotics was not concentration-dependent over the range of 0·01–1·0 mM, therefore it seems unlikely that these drugs are absorbed by specialized transport mechanisms. As is evident from Table 1, the partition coefficients of these antibiotics were small, and no correlation was observed between the partition properties and the absorption rate constants. This means that the absorption characteristics cannot be explained by the lipid solubility on the pH-partition hypothesis.

To elucidate the absorption characteristics of the antibiotics, their interaction with the mucosal lipids was examined. However, drug transfer to the chloroform containing the total lipid extracts of the intestinal mucosa (1 mg ml^{-1}) was small, and there was no correlation with absorbability, therefore interaction between the antibiotics and the lipid components in the plasma membrane seems not to be an important factor in their intestinal absorption.

Permeability of drugs across liposomal membranes

The transfer rate of cephalexin across the liposomal membrane is markedly faster than that of cefazolin and the pH-profile of the transfer rate of cephalexin across the lipid bilayers is similar to the pH-absorption profile (Yasuhara et al 1977). In order to investigate the generality, the relation between the release rate of the antibiotics from the aqueous liposomal dispersion and the absorption rate from gut was examined.

The release rate of the antibiotics from the Visking dialysis sac was rapid, therefore transport across the lipid bilayer of the liposomes could be taken as the rate-limiting process of release to the external medium. A semilogarithmic plot of the percentage of drug remained in the dialysis bag against time gave a straight line and the release rate constants were determined from the slopes of these plots and are listed in Table 2. Those from intestinal lipid liposomes are faster than those from egg lecithin liposomes, and these values are approximately in the rank order of absorbability. Better correlation was observed with the intestinal lipid liposomes. The plot of the release rate constants from intestinal lipid liposomes against the absorption rate constants is shown in Fig. 1. The absorption rate constant for amoxicillin is that when the tissue was pretreated with $HgCl_2$. As is evident from Fig. 1, there is good correlation except for cephalothin and ampicillin. The regression line had a correlation coefficient of 0·988. This means that the intestinal absorption of β-lactam antibiotics cannot be explained by their lipid solubility but rather by the permeability of lipid bilayer membranes in the brush border mem-

Table 2. Overall release rate constants of β-lactam antibiotics from egg lecithin liposomes and intestinal lipid liposomes. Egg lecithin liposome was composed of egg phosphatidylcholine (80 μmol), cholesterol (20 μmol), and dicetylphosphate (5 μmol), and intestinal lipid liposome was from the total lipid extracts from the intestinal mucosa (32 mg). Drug release from the drug-entrapped liposome was determined by a dynamic dialysis method at 37 °C.

Drug	Release rate constant ($\times 10^{-4}$ min^{-1})* Egg lecithin liposome	Intestinal lipid liposome
Ampicillin	69·0 (32·7)	211·9 (27·2)
Amoxicillin	2·7 (0·4)	59·3 (0·2)
Cephalothin	106·3 (0·6)	186·5 (4·9)
Cephaloridine	2·2 (0·6)	45·1 (6·8)
Cephradine	99·6 (4·0)	187·9 (20·9)
Cephalexin	109·6 (8·6)	112·9 (6·6)
Ceftezol	8·4 (0·8)	68·0 (3·6)
Cefazolin	3·8 (0·2)	51·6(7·3)

* All measurements were carried out in duplicate and represent the mean (with s.d.).

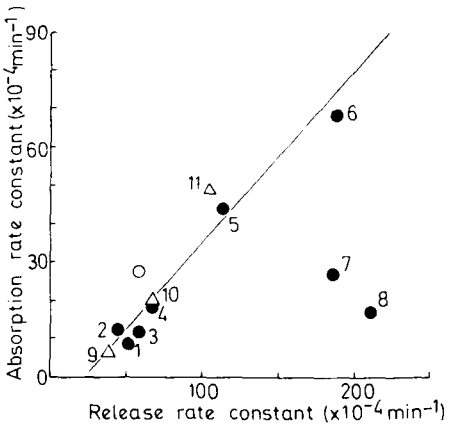

FIG. 1. Plot of overall release rate constants from intestinal lipid liposomes against absorption rate constants for β-lactam antibiotics. ●: 1, cefazolin; 2, cephaloridine; 3, amoxicillin ($HgCl_2$-treated); 4, ceftezol; 5, cephalexin; 6, cephradine; 7, cephalothin; 8, ampicillin. ○: amoxicillin (untreated). △: 9, phenol red; 10, bromphenol blue; 11, quinine.

brane, that is, the plasma membrane is not a simple hydrophobic boundary, but should be a lipid bilayer membrane. Better correlation with intestinal lipid liposomes suggests that some of the lipid components, such as glycolipids and free fatty acids, not present in the egg lecithin liposomes, control the transfer of the antibiotics across the plasma membranes.

In Fig. 1, two kinds of deviation from the regression line were noted; first, the absorption of amoxicillin from the untreated intestine is faster than would be estimated from the regression line, which supports carrier-mediated absorption of this antibiotic; and second, the release rate constants for cephalothin and amoxicillin are much faster than those on the line. To clarify this anomalous permeation behaviour across the lipid bilayer, the temperature dependence of the release rate constant was examined. The Arrhenius plot gave a straight line in the range of 21–37 °C. The activation energies were calculated from the slopes and are listed in Table 3. The activation energies for cephalothin and ampicillin

Table 3. Activation energies for the overall release of β-lactam antibiotics from intestinal lipid liposomes. Drug release from intestinal lipid liposomes was examined at 21, 26, 31, and 37 °C. Activation energies were calculated from the slope of an Arrhenius plot and are given (with s.d.).

Drug	Activation energy (kcal mol^{-1})	(kJ mol^{-1})
Ampicillin	11·9 (3·0)	49·8 (12·5)
Cephalothin	11·9 (2·9)	49·8 (12·1)
Cephradine	16·5 (2·4)	69·0 (10·0)
Cephalexin	17·7 (1·5)	74·0 (6·3)

were smaller than those for cephradine and cephalexin. The relationship between various membrane functions and membrane fluidity has been studied using an Arrhenius plot by Block et al (1977), who suggested that activation energies are closely related to the membrane fluidity. Accordingly, it may be considered from the smaller activation energies of cephalothin and ampicillin that the fluidity or the permeability of the lipid bilayer is influenced by them. To investigate this, the effect of cephalothin on the permeability of liposomes was examined. Fig. 2 shows the release of glucose from total lipid liposomes in the presence of cephalothin. The release rate constants of glucose with or without cephalothin

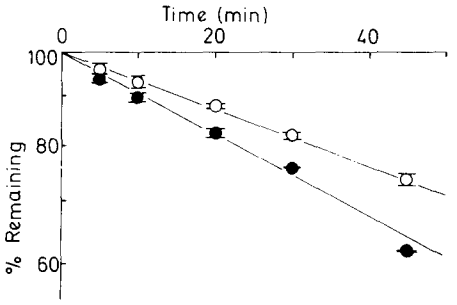

FIG. 2. Effect of cephalothin on the overall release rate of glucose from intestinal lipid liposomes. 20 mM cephalothin was added at the time of preparing liposomes. ○: control. ●: in the presence of cephalothin. Vertical bars represent the mean with s.d. of duplicate experiments.

were $10{\cdot}2 \times 10^{-3}$ min^{-1} and $6{\cdot}6 \times 10^{-3}$ min^{-1}, respectively. This result indicates that the permeability of the lipid bilayer is increased by cephalothin.

One of the possible physicochemical properties of drugs affecting the lipid bilayer could be their surface activity. Fig. 3 shows the surface tension values of the β-lactam antibiotics in aqueous solution at various concentrations at pH 6·5. The values were similar within the concentration range of 0·01–1·0 mM, but at higher concentrations, where liposomes were prepared, the extents of the surface tension lowering by cephalothin and ampicillin were

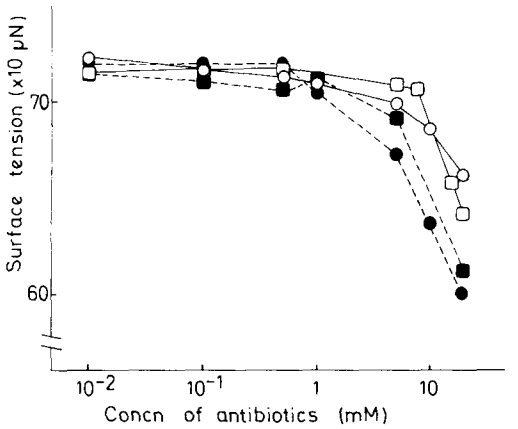

FIG. 3. Surface tension of β-lactam antibiotics in aqueous solution at various concentrations. Drugs are dissolved in pH 6·5 isotonic sodium phosphate buffer and the surface tension was measured at 25 °C, using a Du Noüy Tensiometer. Surface tension of pH 6·5 phosphate buffer was 72·8 mN m^{-1}. □: cefazolin. ○: cephalexin. ■ cephalothin. ●: ampicillin.

larger than those by cephalexin and cefazolin. From these results, it seems reasonable to consider that the higher release rates than expected for cephalothin and ampicillin are caused by their own permeability-increasing effects on the liposomal lipid bilayer, probably due to their surface activity. However, such an effect should not be observed in the case of absorption experiments because of their lower concentration used. Furthermore, the structure of intestinal brush border membrane consists of not only a lipid bilayer but also membrane protein, glycocalyx, and surface mucous layer. Such a complex structure may prevent the direct action of these two compounds on the lipid bilayer membrane.

To investigate whether liposomes are generally applicable as a model for the intestinal absorption of drugs, three other substances, phenol red, bromphenol blue, and quinine, having different degrees of absorbability were tested. As shown in Fig. 1, the findings fitted the regression line for the β-lactam antibiotics suggesting an applicability to general drugs, although there are exceptions.

Liposomes can be easily prepared by using the appropriate lipids and the structures are stable. Furthermore, water-soluble drugs can be entrapped in their aqueous phase, and are released according to apparent first order kinetics. Although the dynamic dialysis method is only applicable to drugs having low permeability, i.e. transfer across the dialysis bag is not rate-limiting, liposomes have potential as a model for assessing the intestinal absorption of drugs.

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