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Comparison of Transport Characteristics of Amino β-Lactam Antibiotics and Dipeptides Across Rat Intestinal Brush Border Membrane

KEN ISEKI, MITSURU SUGAWARA, HIROSHI SAITOH, KATSUMI MIYAZAKI AND TAKAICHI ARITA*

*Department of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo 060 and *Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 11-16, Koshien Kyuban-cho, Nishinomiya, Hyogo 663, Japan*

Abstract—The transport characteristics of amino β-lactam antibiotics, ampicillin and cephradine, have been examined and compared with that of glycylglycine using brush border membrane vesicles isolated from rat small intestine. The initial rate of glycylglycine uptake was markedly stimulated in the presence of an inward H^+ gradient compared with the uptake rates in the absence of an H^+ gradient. With the same H^+ gradient the stimulation of cephradine uptake was lower and ampicillin uptake was not altered. Cephradine uptake, however, was greater than that of glycylglycine in both vesicular conditions ($(pH)_i > (pH)_o$ and $(pH)_i = (pH)_o$). Inhibitory effects of dipeptides, ampicillin and cephradine on the initial uptake of glycylglycine were also examined. Glycylglycine uptake was significantly decreased in the presence of L-phenylalanylglycine or carnosine. Ampicillin and cephradine did not alter the uptake of glycylglycine. These results suggest that the contribution of the inward H^+ gradient to the permeation of ampicillin, cephradine and glycylglycine across the rat small intestinal brush border membranes is different for each of the substances examined.

It is well known that amino β-lactam antibiotics are efficiently absorbed from the intestinal lumen after oral administration, although they are completely ionized over the pH range in the gastrointestinal tract and generally have poor lipid solubility. Therefore, the mechanisms of intestinal absorption of these drugs have been extensively investigated. Some reports (Nakashima et al 1984; Kimura et al 1985; Okano et al 1986a,b) suggested that a carrier-mediated transport system for dipeptides, which have a zwitterionic structure like these drugs, participates in the membrane transport of several amino β-lactam antibiotics. On the other hand, in our previous report (Iseki et al 1988) dealing with the effect of chlorpromazine on the membrane transport of these drugs and endogenous compounds, it was suggested that the permeation characteristics of the amino β-lactam antibiotics (ampicillin, cephradine and cephalexin) differ from those of actively transported substances (glycylglycine and D-glucose). We also found a soluble protein fraction which binds amino β-lactam antibiotics in rat intestinal epithelial cells, and indicated that this intracellular binding process played a role in the absorption of these zwitterionic penicillins and cephalosporins (Miyazaki et al 1982; Iseki et al 1987a,b). Thus, it is considered that the absorption mechanisms of amino β-lactam antibiotics are complicated and there remains controversy about the extent to which the carrier-mediated transport systems participate in the absorption process.

Moreover, in most reports using brush border membrane vesicles from epithelial cells of small intestine, the transport characteristics of amino β-lactam antibiotics have been examined in relatively high drug concentrations (above 1 mM) because of the lack of sensitivity of the assay method, although the specific transport mechanisms of many nutrients, i.e. permeation characteristics driven by ion gradient or H^+ gradient, have been revealed at the lower substrate concentrations (below 50 μM). Furthermore, species differences in the transport characteristics of these amino β-lactam antibiotics have been reported. Okano et al (1986a,b) pointed out that the effects of an inward H^+ gradient on the uptake behaviour of cephradine across the brush border membrane from the rabbit intestine are much larger than that from rat intestine. Muranushi et al (1987) reported that 7432-S, a new orally-active aminocephalosporin, is well absorbed in man, dogs and rats and slightly absorbed in rabbits and monkeys. In our previous reports (Miyazaki et al 1977, 1979; Umeniwa et al 1979) which compared the degree of absorption of ampicillin, amoxycillin, cephalexin and cephradine, good correlation of human and rat results was observed. Despite many investigations, the common properties of the transport system across the brush border membrane of epithelial cells of these aminopenicillins and aminocephalosporins, remain unclear.

To clarify the absorption mechanisms of amino β-lactam antibiotics, in the present study we have compared the transport characteristics of ampicillin, cephradine and glycylglycine at a concentration of 50 μM, i.e. assessed the effect of an inward H^+ gradient of membrane permeation and the effect of dipeptides and these antibiotics on the glycylglycine uptake, using the brush border membrane vesicles isolated from rat small intestine.

Correspondence to: K. Miyazaki, Dept. of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo 060, Japan.

Materials and Methods

Materials

Ampicillin anhydrous (Takeda Chemical Industries, Osaka, Japan), cephradine (Sankyo Co., Tokyo, Japan) were kindly donated. L-[U-^{14}C]Alanine (sp. act. 165 mCi mmol^{-1}) and [U-^{14}C]glycylglycine (sp. act. 100 mCi mmol^{-1}) were purchased from Amersham International Ltd (Bucks, UK). All

other chemicals were of the highest grade available and used without further purification.

Preparation of the brush border membrane vesicles

The entire small intestine of male Wistar rats (180-230g) was excised under anaesthesia with diethyl ether and brush border membrane vesicles were isolated according to the calcium chloride precipitation method of Kessler et al (1978). All steps were performed on ice or at 4°C. The intestine was washed with ice-cold saline and cut longitudinally. The mucosa was scraped gently with a glass microscope slide. The scrapings (4 g wet weight) were homogenized with a Waring blender (Nihonseiki, Japan) at 16 500 rev min^{-1} for 4 min in 100 mL of 50 mM D-mannitol, 2 mM Tris-HCl, pH 7·1. $CaCl_2$ solution (0·5 M) was added to a final concentration of 10 mM, and the homogenate was allowed to stand for 20 min. The homogenate was centrifuged at 3000 *g* for 15 min in a Hitachi high speed refrigerated centrifuge 20 PR-52 (rotor RPR 20-2). The supernatant was then centrifuged at 27 000 *g* for 30 min. The resulting pellet was suspended in 40 mL of the buffer needed for the experiment (experimental buffer), and homogenized in a glass/Teflon Dounce-type homogenizer with 10 strokes. After a final centrifugation of 27 000 *g* for 30 min, brush border membranes were suspended in the experimental buffer with a Dounce-type homogenizer. Experimental buffers were: 20 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethansulphonic acid (Hepes)/Tris and 100 mM D-mannitol, pH 7·5 (buffer A), 20 mM 2-(*N*-morpholino) ethansulphonic acid (Mes)/Tris, 100 mM KCl, and 100 mM D-mannitol, pH 6·0 (buffer B), and 20 mM Hepes/Tris, 100 mM KCl, and 100 mM D-mannitol, pH 7·5 (buffer C). In other experiments examining the effect of a protease inhibitor on the uptake of glycylglycine, phenylmethylsulphonyl fluoride (PMSF, 0·1 mM) was added to the medium for the isolation and suspension of the vesicles and the substrate solution. Brush border membrane vesicles were used for transport studies within 4 h of preparation.

The purity of the membrane was routinely evaluated by the enrichment of alkaline phosphatase (E.C.3.1.3.1.), an enzyme specific to the intestinal brush border membrane. The specific activity of this enzyme increased above 12-fold in the final membrane suspension compared with concentrations found in the homogenate of intestinal scraping. The membrane was equilibrated for 1 h at 0°C before use.

Transport studies

The uptake of substrates was measured by a rapid filtration technique. In the regular assay, the reaction was initiated by the addition of 100 μL of an experimental buffer containing the substrate to 20 μL of membrane suspension (approximately 6 mg of protein mL^{-1}) at 25°C.

At a stated time the reaction was stopped by diluting the reaction mixture with 5 mL of ice-cold buffer (150 mM NaCl, 20 mM Hepes/Tris, pH 7·5). The tube contents were immediately filtered through a Millipore filter (HAWP, 0·45 μm, 2·5 cm diameter) which was washed once with 8 mL of the same ice-cold buffer.

In a separate experiment, non-specific adsorption onto the Millipore filter was determined using the incubation medium instead of the brush border membrane suspension. This value was subtracted from the uptake data.

Analytical methods

L-[U-^{14}C]Alanine or [U-^{14}]glycylglycine on the filter was determined by liquid scintillation counting. Ampicillin and cephradine were determined by high-performance liquid chromatography using fluorometric detection (Miyazaki et al 1983). Protein concentrations were determined by the method of Lowry et al (1951) with bovine serum albumin as standard.

Results

Transport ability of isolated vesicles for L-alanine

To examine whether the isolated vesicles have an intact transport property, the uphill transport of L-alanine, an actively transported substance under an inward Na^+ gradient, was measured in the presence of Na^+ or K^+ gradient. As shown in Fig. 1, the initial rate of L-alanine uptake was markedly stimulated in the presence of an inward Na^+ gradient and an apparent overshoot phenomenon was observed. Furthermore, the effect of extravesicular osmolarity on L-alanine uptake at steady state (30 min) was measured using D-cellobiose (100-300 mM) as the impermeant solute. The uptake of L-alanine was inversely proportional to the extravesicular osmolarity and extrapolation to infinite extravesicular osmolarity (zero intravesicular space) was negligible compared with uptake of L-alanine into the brush border membrane vesicles (data not shown). These results suggest that the brush border membrane vesicles prepared in this study have intact transport properties.

Effect of an inward H^+ gradient on the time course of glycylglycine, cephradine and ampicillin

The uptake of glycylglycine, cephradine and ampicillin by brush border membrane vesicles was measured in the presence and absence of an inward H^+ gradient. As shown in Fig. 2, the initial rate of glycylglycine uptake (0·5 and 1·0 min) was markedly stimulated in the presence of an inward

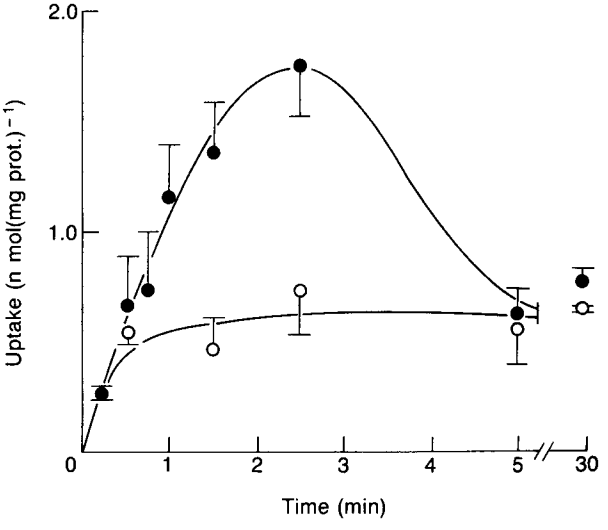

FIG. 1. Uptake of L-alanine by rat intestinal brush border membrane vesicles. Membrane vesicles were preincubated at 25°C in buffer A for 2 min. The vesicles (20 μL) were incubated with buffer A (100 μL) containing 120 mM NaCl (●) or 120 mM KCl (○) and 1·2 mM L-alanine at 25°C. Each point represents the mean ± s.e.m. of 3-6 measurements with different preparations of vesicles.

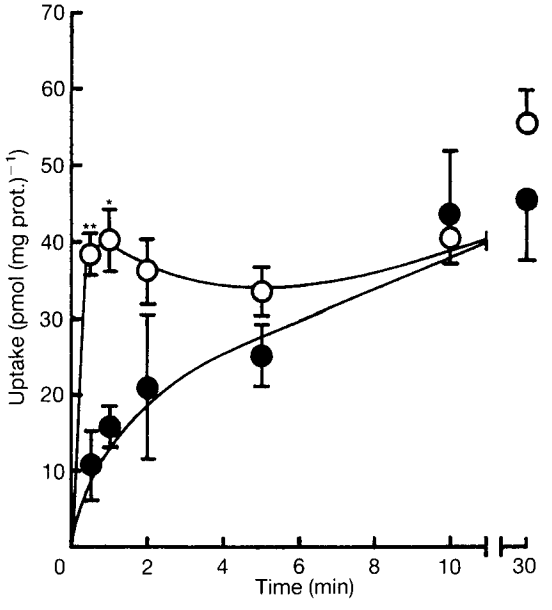

FIG. 2. Effect of H^+ gradient on glycylglycine uptake by rat intestinal brush border membrane vesicles. Membrane vesicles were preincubated at 25°C in buffer B (●) or buffer C (○) for 2 min. The vesicles (20 μL) were incubated with buffer B (100 μL) containing 60 μM glycylglycine at 25°C. Each point represents the mean ± s.e.m. of 4-11 measurements with different preparations of vesicles. $*P < 0.025$, $**P < 0.001$, significantly different from no H^+ gradient.

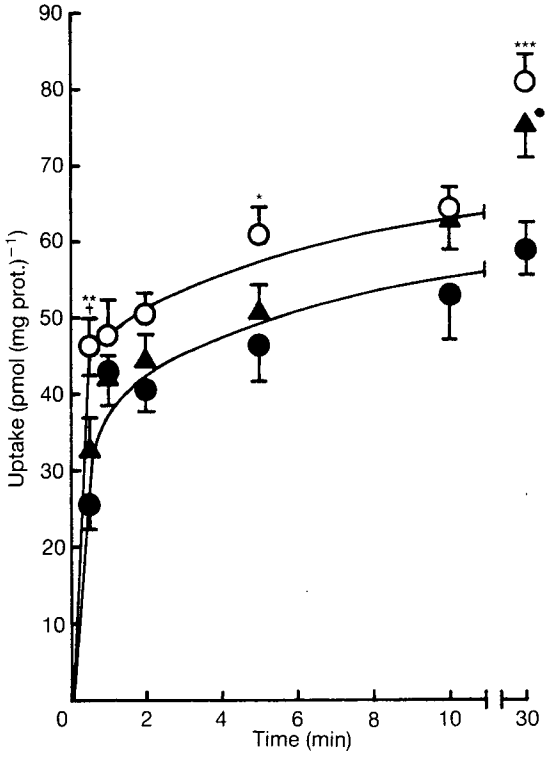

FIG. 3. Effect of H^+ gradient on cephradine uptake by rat intestinal brush border membrane vesicles. Membrane vesicles were preincubated at 25°C in buffer B (●) or buffer C (○, ▲) for 2 min. The vesicles (20 μL) were incubated with buffer B (100 μL) (○, ●) or buffer C (100 μL) (▲) containing 60 μM cephradine at 25°C. Each point represents the mean ± s.e.m. of 4-10 measurements with different preparations of vesicles. $*P < 0.05$, $**P < 0.025$, $***P < 0.001$, significantly different from no H^+ gradient (pH 6·0). $\dagger P < 0.05$, significantly different from no H^+ gradient (pH 7·5). $\bullet P < 0.05$, significantly different from no H^+ gradient (pH 6·0).

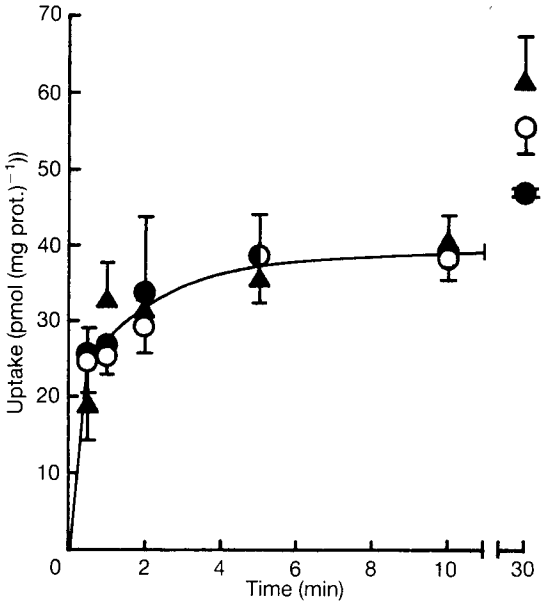

FIG. 4. Effect of H^+ gradient on ampicillin uptake by rat inestinal brush border membrane vesicles. Membrane vesicles were preincubated at 25°C in buffer B (●) or buffer C (○, ▲) for 2 min. The vesicles (20 μL) were incubated with buffer B (100 μL) (○, ●) or buffer C (100 μL) (▲) containing 60 μM ampicillin at 25°C. Each point represents the mean ± s.e.m. of 4-15 measurements with different preparations of vesicles. In the results at 30 min, when equality of variances estimated by F test was not obtained, the Cochran-Cox method was used to estimate statistical differences instead of the *t*-test. There was no significant difference between the presence (○) and absence (●) of H^+ gradient, and also between medium pH 6·0 (●) and medium pH 7·5 (▲). Moreover, there was no significant difference between the presence (○) and absence (▲) of H^+ gradient by the *t*-test.

H^+ gradient ($(pH)_i = 7.5, (pH)_o = 6.0$) compared with the uptake rates in the absence of H^+ gradient ($(pH)_i = (pH)_o = 6.0$). As shown in Fig. 3, the stimulation of cephradine uptake by an H^+ gradient, on the contrary, was lower, although both the time course of uptake in the presence and absence of an H^+ gradient were larger than those of glycylglycine. For ampicillin, as shown in Fig. 4, there was no change in the time course of uptake among three vesicular conditions ($(pH)_i > (pH)_o$, $(pH)_i = (pH)_o = 6.0$ or 7·5). The ratio of initial uptake (30 s) in the presence and absence ($(pH)_i = (pH)_o = 6.0$) of H^+ gradient for glycylglycine, cephradine and ampicillin was 3·6, 1·7, and 1·0, respectively.

In the glycylglycine uptake study in the presence of an H^+ gradient, each time course of uptake in the presence and absence of the protease inhibitor, PMSF, was almost the same (data not shown).

Adsorption of ampicillin and cephradine to the brush border membrane vesicles

To examine whether the higher uptake of cephradine compared with that of ampicillin was due to the adsorption to the brush border membrane, the effect of extravesicular osomolarity on ampicillin and cephradine uptake at steady-state (60 min) was investigated in the presence of an inward H^+ gradient. As shown in Fig. 5, the uptake of both antibiotics was inversely proportional to the extravesicular osmolarity. Each extrapolation space was negligible, −3·1

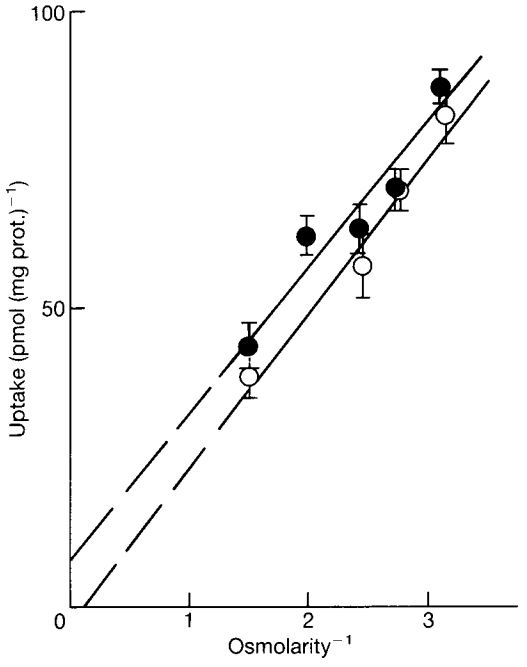

FIG. 5. Effect of medium osmolarity on ampicillin and cephradine uptake by rat intestinal brush border membrane vesicles. Membrane vesicles were suspended in buffer C. The vesicles (20 μL) were incubated with buffer B (100 μL) containing 60 μM ampicillin (○) or cephradine (●) and various concentrations of D-cellobiose at 25°C for 60 min. Each point represents the mean±s.e.m. of four measurements.

pmol mg protein^{-1} for ampicillin and 8·5 pmol mg protein^{-1} for cephradine, compared with each uptake of the antibiotics in the brush border membrane vesicles. This result and the initial uptake data (Fig. 3) suggest that the permeation of cephradine across the membrane is comparatively rapid.

Effect of dipeptides and antibiotics on glycylglycine uptake by brush border membrane vesicles

To further elucidate the difference of permeation characteristics between dipeptides and amino β-lactam antibiotics, the effect of dipeptides, ampicillin and cephradine on the initial uptake of glycylglycine (30 s) by the brush border membrane vesicles was investigated in the presence of an inward H^+ gradient. As shown in Table 1, glycylglycine uptake was significantly inhibited in the presence of 2·5 mM L-phenylalanylglycine or carnosine. On the contrary, ampicillin and cephradine did not alter the uptake of glycylglycine.

Table 1. Effect of dipeptides and amino-β-lactam antibiotics on glycylglycine uptake by rat intestinal brush border membrane vesicles

Inhibitor	Concn (mM)	Glycylglycine uptake pmol mg prot.$^{-1}$ per 30s	%
None		32·3±2·2	100
L-Phenylalanylglycine	1·0	24·9±3·3	77
	2·5	21·9±4·3*	68
L-Carnosine	1·0	28·7±4·6	89
	2·5	22·4±1·9**	69
Ampicillin	1·0	33·9±2·3	105
	2·5	28·6±1·1	89
Cephradine	1·0	31·4±2·1	97
	2·5	27·3±2·5	85

Membrane vesicles were preincubated at 25°C in buffer B for 2 min. The vesicles (20 μL) were incubated with buffer A (100 μL) containing glycylglycine (60 μM) and inhibitor (1·2 or 3 mM) at 25°C for 30 s. Each value represents the mean s.e.m. of 4–10 measurements with different preparations of vesicles. *$P>0·05$, **$P>0·025$, significantly different from control.

Discussion

In this study we have compared the transport characteristics of glycylglycine and amino β-lactam antibiotics using the rat small intestinal brush border membrane vesicles. We confirmed that isolated vesicles have an intact transport ability using L-alanine as an actively transported substrate. The initial rate of glycylglycine uptake (0·5 and 1·0 min) was markedly stimulated in the presence of an inward H^+ gradient, although the obvious overshoot was not obtained (Fig. 2). In the same conditions, on the contrary, the stimulation of cephradine uptake was lower while ampicillin uptake was not altered (Figs 3, 4). In addition, the tendency for overshoot was not observed within these drugs, and both time courses of cephradine uptake in the presence and absence of an H^+ gradient were greater than those of glycylglycine. Moreover, the initial uptake of glycylglycine (30 s) was significantly inhibited by dipeptides (L-phenylalanylglycine and carnosine), but not antibiotics (Table 1). It was obvious that the acceleration observed in glycylglycine uptake was not due to the uptake of glycine, a degradation product of glycylglycine, since there was no change in the time course of uptake between the vesicles with and without the protease inhibitor. Also, the uptake study was carried out in the absence of sodium ions which are known to stimulate amino acid uptake. Recent studies with brush border membrane vesicles from the small intestine have suggested that an inward H^+ gradient is the driving force for the transport of several dipeptides (Ganapathy & Leibach 1985; Hoshi 1985; Ferraris et al 1988; Said et al 1988). However, there are few reports with direct evidence (overshoot phenomenon) for H^+ coupled dipeptide transport in the rat small intestinal brush border membrane vesicles (Said et al 1988). In our present study, the evidence of the overshoot phenomenon was observed within the first 5 min (0·5-5 min), thereafter, the uptake increased gradually. The reason for the uptake difference between L-amino acids and dipeptides in the rat small intestine remains unclear.

It has been pointed out that the uptake rate of several aminocephalosporins by the brush border membrane from rabbit and rat small intestine was stimulated in the presence of an inward H^+ gradient (Okano et al 1986a, b) and that the degree of stimulation in the rat intestinal preparation was much lower compared with that of rabbit (Okano et al 1986a,b). Our results in the present study (Fig. 3) are consistent with those of Okano et al (1986a) in that cephradine uptake was stimulated slightly in the presence of an inward H^+ gradient, but those authors did not study directly the dipeptide uptake; they also used a relatively higher drug concentration (1 mM). In the present experiment, we studied a lower substrate concentration (50 μM) to compare precisely the transport characteristics of drug and dipeptide. It was observed that the extents of the contribu-

tion of inward H^+ gradient differ among ampicillin, cephradine and glycylglycine. Moreover, the uptake rate of cephradine was greater than that of glycylglycine in the absence of an H^+ gradient (Figs 2, 3). From the results, it might be suggested that these drugs, especially ampicillin, have a transport system independent of H^+-dependent carrier mediated transport mechanisms. The degree of the participation of the H^+-dependent carrier mediated system in cephradine uptake in the rat small intestine needs further examination. Our present results are in good agreement with those in our previous report (Iseki et al 1988) suggesting the difference in the transport properties between the amino β-lactam antibiotics (ampicillin, cephradine and cephalexin) and actively transported substances (glycylglycine and D-glucose).

Although the problems of species differences should be further examined, it has been reported that the degree of absorption of several β-lactam antibiotics in human and rat are well correlated (Miyazaki et al 1977, 1979; Umeniwa et al 1979; Muranushi et al 1987).

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