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Report

Transport Characteristics of Ceftibuten, a New Oral Cephem, in Rat Intestinal Brush-Border Membrane Vesicles: Relationship to Oligopeptide and Amino β-Lactam Transport

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Ceftibuten undergoes H^+-coupled uphill transport across rat small intestinal brush-border membrane vesicles. The effects of amino acids, peptides, folate, and β-lactams on the uptake of ceftibuten were examined. Uptake of ceftibuten was competitively inhibited by dipeptides or tripeptides. A countertransport effect on ceftibuten uptake was observed in the vesicle preloaded with these peptides, and the transport was temporarily against a concentration gradient (overshooting). On the other hand, ceftibuten uptake was not changed by amino acids and a tetrapeptide. Therefore, ceftibuten is predominantly transported via the oligopeptide transport system in the brush-border membranes. The relationship of ceftibuten transport to folate and other oral antibiotics was also investigated. Cyclacillin, cephradine, and cefadroxil exhibited both inhibitory and countertransport effects, but folate, cefaclor, and cephalexin showed only a slight inhibitory effect. As the transport of cefaclor showed no uphill uptake in the presence of a H^+ gradient and its H^+ stimulated uptake was small, a H^+ gradient-independent carrier-mediated system seems to participate in its transport. These findings suggest that two different carrier-mediated transport systems, H^+ gradient dependent and independent, may exist for oral cephems.

KEY WORDS: ceftibuten; transport; brush-border membrane; oligopeptide; amino β-lactam; oral cephem.

INTRODUCTION

Ceftibuten, (6*R*,7*R*)-7-[(Z)-2-(2-aminothiazol-4-yl)-4-carboxy-2-butenoylamino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (Fig. 1), is a new orally active cephalosporin (1). It shows excellent bioavailability in humans (2) and animals (3), although it does not possess an α-amino group in the side chain at the 7 position of the cephem skeleton, which distinguishes it from conventional oral amino β-lactams. We have previously demonstrated (4) that ceftibuten was actively transported in rat intestinal brush-border membrane with a H^+ gradient as a driving force which led to efficient intestinal absorbability. The present investigation was carried out to elucidate further the transport mechanism of ceftibuten by using rat intestinal brush-border membrane vesicles.

In recent years, transport studies with purified intestinal brush-border membrane vesicles have revealed the carrier-mediated transport mechanisms of various compounds. The Na^+ gradient across the apical membrane is an energy source of the transport of D-glucose (5), amino acids (6), and bile acid (7), while a H^+ gradient serves as a driving force in the transport of dipeptides (8,9) and folate (10). These techniques were also applied to study the transport of amino β-lactams (11–14), and Okano *et al.* have reported that amino β-lactams are transported via the dipeptide transport system (13,14).

In view of the similar driving force in their transport, the inhibitory and countertransport effects of oligopeptides, folate, and amino β-lactams on the vesicle uptake of ceftibuten were evaluated.

MATERIALS AND METHODS

Materials

Ceftibuten and latamoxef were used as obtained from Shionogi Research Laboratories. Cefaclor and cephalexin were supplied from Eli Lilly (Indianapolis, Ind.). Cephradine was purchased from Sankyo Co. (Tokyo), cyclacillin from Takeda Chemical Industries (Osaka, Japan), cefadroxil and ampicillin from Sigma Chemical Co. (St. Louis, Mo.), and cefazolin and ceftizoxime from Fujisawa Pharmaceutical Co. (Osaka, Japan). γ-L-Glutamyl-L-alanyl-L-alanine and γ-L-glutamyl-L-alanine were obtained from Bachem (Bubendorf, Switzerland). All other peptides used were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

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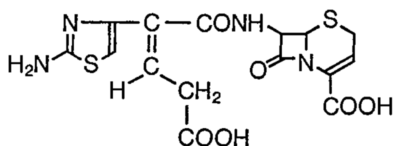

Fig. 1. Chemical structure of ceftibuten.

Preparation of Brush-Border Membrane Vesicles

Brush-border membrane vesicles were isolated from rat small intestine (Jcl SD male, 200–250 g) by the Ca^{2+}-precipitation method of Kessler *et al.* (15) as described previously (4).

Transport Studies

Uptake studies were carried out at 25°C by the rapid filtration technique as described previously (4). The freshly isolated vesicles were resuspended with 10 m*M* Hepes buffer (pH 7.5) containing 100 m*M* mannitol, 100 m*M* KCl and made up to a final concentration of 8–10 mg protein/ml. The vesicle suspension (20 μl) was added to 200 μl of a reaction mixture containing 0.5 m*M* ceftibuten, 100 m*M* mannitol, 100 m*M* KCl, and 10 m*M* Mes (pH 5.5) at 25°C to start the transport experiment. The inhibitory effect on ceftibuten uptake was examined by the addition of various compounds in the reaction solution to a concentration of 10 m*M*. To observe the countertransport effect, membrane vesicles were resuspended with pH 5.5 buffer solution to a final concentration of about 20 mg protein/ml, a portion of the vesicle suspension (10 μl) was preloaded with 10 μl of pH 5.5 buffer solution containing various compounds (20 m*M*) for 5 min at 25°C, then the uptake study was carried out as described above.

Analytical Method

Ceftibuten and cefaclor were measured with a high-performance liquid chromatograph LC-6A (Shimadzu Co., Kyoto, Japan). The conditions were as follows: column, Nucleosil ${}_{10}C_{18}$ for ceftibuten and ${}_{7}C_{18}$ for cefaclor, 4 mm × 30 cm (Macherey-Nagel, Germany); mobile phase, 0.1 *M* ammonium acetate/methanol = 12/1 for ceftibuten and 0.1 *M* ammonium acetate/acetonitrile = 88/12 for cefaclor; flow rate, 1.0 ml/min; and wavelength, 262 nm for both drugs. Protein was assayed by the Bio-Rad protein assay kit (Bio-Rad, Richmond, Calif.) with bovine γ-globulin as a standard.

RESULTS

Inhibitory Effect of Amino Acids and Oligopeptides

As shown in the preceding paper (4), the uptake of ceftibuten was linear for the initial 15 sec under an inward H^+ gradient; thus, the uptake over 15 sec was used to represent the initial uptake. The effects of amino acids and oligopeptides (10 m*M*) on the initial uptake of ceftibuten in the presence of a H^+ gradient are shown in Fig. 2. Amino acids (glycine and L-alanine) and a tetrapeptide (glycylglyclyglycylglycine) had no effect, while dipeptides and tripeptides, except for D-alanyl-D-alanine, strongly inhibited the uptake of ceftibuten. These data indicated that the transport system of oligopeptide played an important role in the transport of ceftibuten.

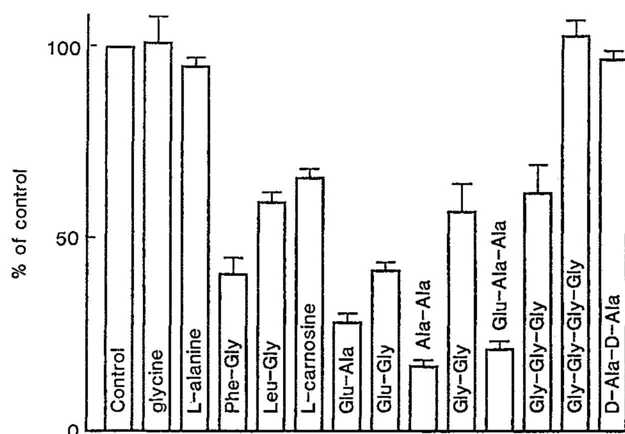

Fig. 2. Effects of amino acids and oligopeptides on ceftibuten uptake by intestinal brush-border membrane vesicles in the presence of H^+ gradient. Control value of the initial uptake is 1.11 ± 0.03 nmol/mg protein/15 sec. Data represent the mean ± SE (N = 3–4).

Kinetic Analyses of the Inhibition by Oligopeptide

The Lineweaver–Burk plot was used to distinguish the type of inhibition. Figures 3 and 4 show the reciprocal of the initial rate of ceftibuten uptake after correction for the nonsaturable component (11) in the presence of γ-L-glutamyl-L-alanine and γ-L-glutamyl-L-alanyl-L-alanine, respectively. All of the lines in both figures intersect nearly at the same point on the vertical axis, indicating that these peptides competitively inhibit ceftibuten uptake. Kinetic parameters of ceftibuten estimated by NONLIN (16) to fit the data to Eq. (1) are as follows: K_m = 0.17 m*M*; V_{max} = 4.72 nmol/mg protein/min; and K_{dif} = 0.30 nmol/mg protein/min/m*M* (4).

$$V = V_{max} \cdot C/(K_m + C) + K_{dif} \cdot C \qquad (1)$$

where V is the initial uptake rate, C is the initial concentration, V_{max} is the maximum uptake rate by the carrier-mediated process, K_m is the Michaelis constant, and K_{dif} is the coefficient of simple diffusion. The mean inhibitory con-

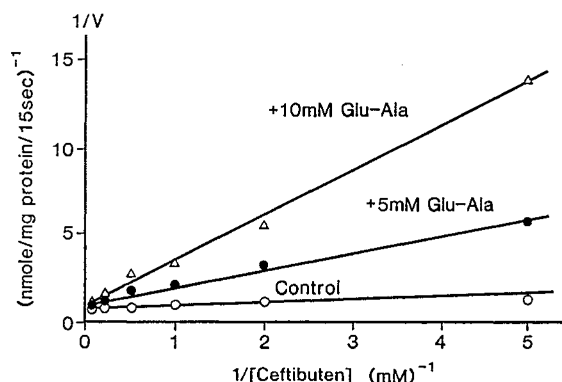

Fig. 3. Lineweaver–Burk plot of ceftibuten uptake in the presence of γ-Glu-Ala. (○) Control; (●) with 5 m*M* γ-Glu-Ala; (△) with 10 m*M* γ-Glu-Ala.

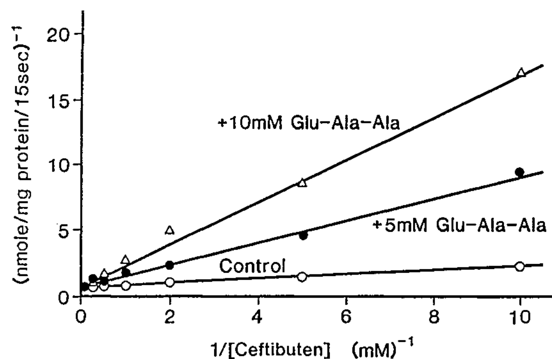

Fig. 4. Lineweaver–Burk plot of ceftibuten uptake in the presence of γ-Glu-Ala-Ala. (○) Control; (●) with 5 mM γ-Glu-Ala-Ala; (△) with 10 mM γ-Glu-Ala-Ala.

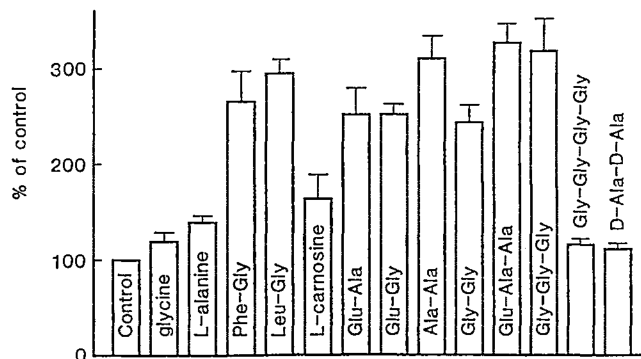

Fig. 6. Countertransport effects of amino acids and oligopeptides on ceftibuten uptake. Control value of ceftibuten uptake is 0.16 ± 0.01 nmol/mg protein/15 sec. Data represent the mean ± SE (N = 4).

stants estimated by fitting the data to Eq. (2) are 0.95 and 1.1 mM for γ-L-glutamyl-L-alanine and γ-L-glutamyl-L-alanyl-L-alanine, respectively:

$$V = V_{\max} \cdot C/[K_m(1 + I/K_i) + C] + K_{\text{dif}} \cdot C \quad (2)$$

where K_i is the inhibitory constant and I is the concentration of the inhibitor.

Countertransport Effects of Amino Acids and Oligopeptides

In order to confirm that ceftibuten is transported by an oligopeptide carrier, the countertransport effects of amino acids and oligopeptides were studied. Figure 5 shows the time course of ceftibuten uptake into vesicles preloaded with glycylglycylglycine and γ-L-glutamyl-L-alanyl-L-alanine. Marked stimulation of ceftibuten uptake was noted which was against the concentration gradient (overshooting). Countertransport effects of the amino acids and oligopeptides tested can be compared in Fig. 6. Although amino acids and a tetrapeptide had no effect, dipeptides and tripeptides, except for D-alanyl-D-alanine, showed significant countertransport effects. These findings support the idea that ceftibuten is transported by an oligopeptide carrier across the small intestinal brush-border membrane.

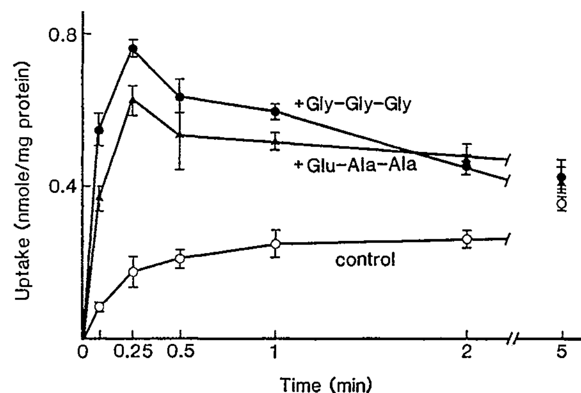

Fig. 5. Time course of ceftibuten uptake into the vesicles preloaded with Gly-Gly-Gly and γ-Glu-Ala-Ala. Data represent the mean ± SE. (○) Control; (●) with 10 mM Gly-Gly-Gly; (▲) with 10 mM γ-Glu-Ala-Ala.

Relation to Folate Transport

The transmembrane H^+ gradient has also been reported to drive folate transport (10). Therefore, we examined the relationship of the transport of ceftibuten to that of folate. The inhibitory effect of folate was relatively small compared with that of oligopeptides, and its countertransport effect was not observed (data not shown). These results show that ceftibuten transport may be independent of folate transport.

Relation to the Transport of Amino β-Lactams

Since carrier-mediated transport mechanism has been reported to participate in the intestinal absorption of amino β-lactams (17–20), the effect of other antibiotics on ceftibuten uptake was studied (Figs. 7 and 9). All of the antibiotics, including those for parenteral use, inhibited ceftibuten uptake more or less, with cyclacillin exhibiting the most extreme inhibition (Fig. 7). The Lineweaver–Burk plot for cyclacillin (Fig. 8) indicates a competitive mode of inhibition with an apparent inhibitory constant of 0.2 mM. Figure 10 shows the countertransport effect of these antibiotics. In the case of cyclacillin, cephradine, and cefadroxil, their preload-

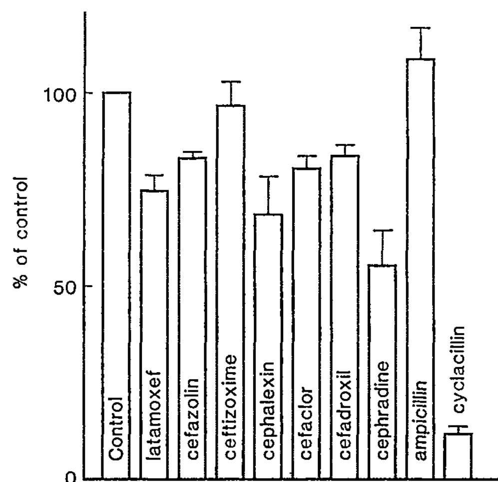

Fig. 7. Effect of various antibiotics on ceftibuten uptake by intestinal brush-border membrane vesicles. Control value of the initial uptake is 1.01 ± 0.04 nmol/mg protein/15 sec. Data represent the mean ± SE (N = 4).

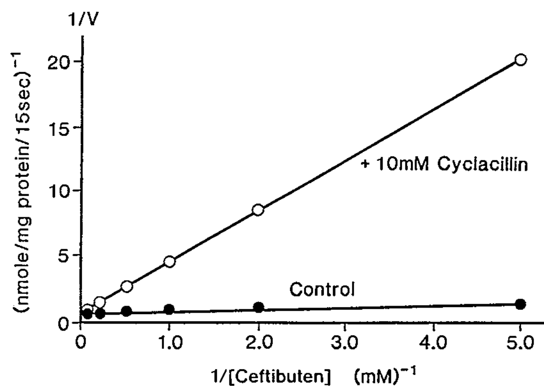

Fig. 8. Lineweaver–Burk plot of ceftibuten uptake in the presence of cyclacillin. (●) Control; (○) with 10 m*M* cyclacillin.

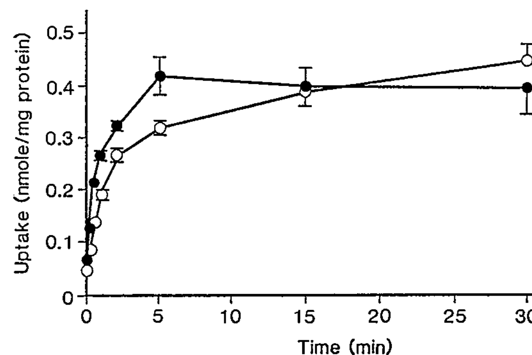

Fig. 10. Effect of H^+ gradient on cefaclor uptake by rat brush-border membrane vesicles in the presence (●) and absence (○) of a H^+ gradient.

ing caused significant stimulation of the initial uptake of ceftibuten. These data suggest the existence of a common carrier for these three antibiotics and ceftibuten. However, cefaclor and cephalexin did not show a marked countertransport effect in spite of their efficient intestinal absorbability. This implies that cefaclor and cephalexin may be transported mainly via other transport systems. To ascertain this, the transport characteristics of cefaclor were also studied. Figure 10 shows the time course of cefaclor uptake in the presence and absence of a H^+ gradient. No overshooting phenomenon was observed even in the presence of a H^+ gradient, and H^+-stimulated uptake was relatively small compared with that for ceftibuten (4).

DISCUSSION

The preceding paper (4) demonstrated that the transport of ceftibuten in the intestinal brush-border membrane vesicles is driven by an inward gradient of H^+ but not Na^+. Recent studies by Ganapathy *et al.* (8) and Takuwa *et al.* (9) showed that the transport of dipeptide across the intestinal brush-border membrane was independent of a Na^+ gradient and energized by a H^+ gradient. Schoron *et al.* (10) reported that folate transport in the jejunum was also driven by a H^+ gradient. Based on these findings, it is reasonable to assume

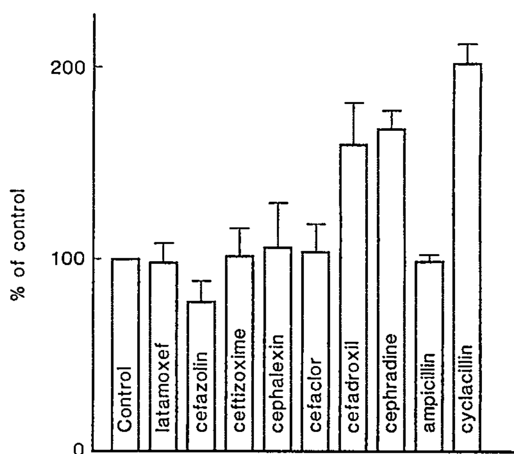

Fig. 9. Countertransport effects of various antibiotics on ceftibuten uptake. Control value of ceftibuten uptake is 0.20 ± 0.01 nmol/mg protein/15 sec. Data represent the mean ± SE (*N* = 4).

that ceftibuten may use the transport system of nutrients, such as dipeptides and folate, with respect to driving force.

Since folate exists as an anion at physiological pH, like ceftibuten, the folate transport system was first presumed to transport ceftibuten. However, folate exhibited only slight effects on ceftibuten uptake. The kinetic parameters of ceftibuten differed greatly from those of folate [K_m = 0.17 m*M*, V_{max} = 4.72 nmol/mg protein/min for ceftibuten (4); K_m = 0.19 μ*M*, V_{max} = 12.8 pmol/mg protein/min for folate (10)]. Therefore, the ceftibuten-transporting carrier is probably not the folate carrier.

In the present study, oligopeptides were found to display significant inhibitory and countertransport effects on ceftibuten uptake (Figs. 2 and 6). These results strongly suggest that ceftibuten is transported by the oligopeptide carrier. We had speculated that ceftibuten might share the transport system of an anionic peptide such as glutamic acid derivatives (e.g., γ-L-glutamyl-L-alanyl-L-alanine); however, zwitter ionic peptides such as glycylglyclyglycine were shown to have an effect similar to that of anionic peptide (Figs. 2 and 6). Since all these compounds are considered to share a common carrier, this transport system is thought to be independent of the overall charge of the substrate. As the transport of ceftibuten was stereoselective (4) and D-alanyl-D-alanine had no effect on ceftibuten uptake, a specific chemical structure seems to be required for this transport system.

The H^+ gradient-stimulated uptake of cefaclor (Fig. 10) was relatively small compared with that of ceftibuten (4). Nevertheless, concentration dependence was seen in the uptake of cefaclor (4) (K_m = 3.0 m*M*, V_{max} = 4.3 nmol/mg protein/min, K_{dif} = 0.16 nmol/mg protein/min/m*M*). Therefore, a carrier-mediated transport system for cefaclor exists which is independent of a H^+ gradient. These findings led to the idea that at least three pathways are available for the uptake of oral antibiotics. One occurs by simple diffusion, the second is a H^+ gradient-dependent carrier-mediated system, and the third is a H^+ gradient-independent carrier-mediated system.

We estimated the contribution of each system to the entire uptake of ceftibuten and cefaclor at 0.5 m*M* for 15 sec in the presence of a H^+ gradient (pH 5.5 outside, pH 7.5 inside). The ratio of the total carrier-mediated transport to the simple diffusion is represented as the ratio of $V_{max}/(K_m$ +

C) to K_{dif} according to Eq. (1), and the contribution of the H^+ gradient-dependent carrier-mediated system is estimated from the difference of drug uptakes in the presence and absence of H^+ gradient at 15 sec. These estimations are summarized in Fig. 11. For ceftibuten, H^+ gradient-dependent carrier-mediated transport is dominant, while for cefaclor it is H^+ gradient-independent carrier-mediated transport. The contribution of the nonsaturable process (simple diffusion) is small for both drugs.

Several investigators have used *in situ* and *in vitro* techniques (17–20) to show that the absorption of amino β-lactams is related to that of dipeptides. Okano *et al.* (13) demonstrated that with rat intestinal brush-border membrane vesicles, cephradine uptake was stimulated in the presence of an inward H^+ gradient, but they did not observe such concentrative uptake as seen with ceftibuten (4). According to our calculation using their kinetic parameters (13) of cephradine (1 m*M*) uptake for 30 sec, the contribution of carrier-mediated transport to the total uptake is 72% in the presence of a H^+ gradient (pH 6.0 outside, pH 7.5 inside) and the H^+ gradient-dependent uptake in the total uptake is estimated to be about 25% from the difference of the uptake in the presence and absence of a H^+ gradient. The lack of concentrative uptake of cephradine may be attributed to the small contribution (about 25%) of the H^+ gradient-dependent uptake. In contrast, in the case of ceftibuten, H^+ gradient-dependent uptake is 87% of the total uptake (Fig. 11), resulting in the pronounced concentrative uptake.

With respect to the effects of antibiotics on ceftibuten uptake, the parenteral cephems such as latamoxef showed an inhibitory effect (Fig. 7) but exhibited no countertransport effect (Fig. 9). These findings imply that the parenteral antibiotics can bind to the carrier but cannot be transported. Both inhibitory and countertransport effects were observed by oral antibiotics, but these effects, except for that of cyclacillin, were smaller than those of dipeptide and the countertransport effects varied among drugs. Since ceftibuten is transported mainly by a H^+ gradient-dependent carrier, the magnitude of the countertransport effect of each drug on ceftibuten uptake is thought to depend on the fraction of H^+ gradient-dependent transport to the total transport of each antibiotic. Cyclacillin exhibited the strongest inhibitory and countertransport effects. Its inhibitory constant is 0.2 m*M*, which is close to the K_m value of ceftibuten (4). Thus, H^+ gradient-dependent transport might contribute predominantly to the transport of cyclacillin.

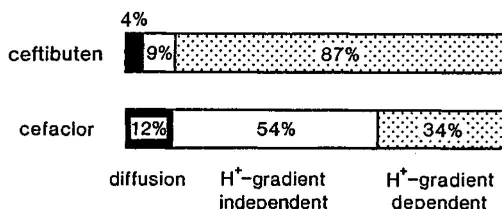

Fig. 11. Contribution of each system on 0.5 m*M* ceftibuten and cefaclor uptake for 15 sec under a H^+ gradient.

Kimura *et al.* proposed two carrier systems to explain their results on the intestinal absorption of cyclacillin with an *in situ* absorption experiment (17). One system is active transport, which can be blocked by dipeptide, and the other is facilitated diffusion, which is inhibited by amoxicillin. Thus, the transport mechanism of cyclacillin seems to be very similar to that of ceftibuten, and its H^+ gradient-dependent transport may correspond to cyclacillin's active transport, while its H^+ gradient-independent transport corresponds to cyclacillin's facilitated diffusion. The contribution of the three absorption processes to drug absorption varies among antibiotics.

The conclusion from the present study is that ceftibuten is absorbed mainly through the oligopeptide transport route in the small intestine, whose driving force is the transmembrane H^+ gradient represented by the existence of an acidic microclimate pH at the surface of the small intestine (21).

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