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Research Article

Transport Characteristics of Ceftibuten (7432-S), a New Oral Cephem, in Rat Intestinal Brush-Border Membrane Vesicles: Proton-Coupled and Stereoselective Transport of Ceftibuten

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The transport characteristics of ceftibuten in rat intestinal brush-border membrane vesicles were investigated by a rapid filtration technique. Ceftibuten uptake was markedly stimulated by an inwardly directed H^+ gradient (pH 7.5 inside, pH 5.5 outside) in comparison with that in the absence of a H^+ gradient. The uptake at 30 sec was four times greater than that observed at equilibrium (overshoot phenomenon), while the H^+ gradient-stimulated uptake of ceftibuten was markedly reduced in the presence of FCCP, a protonophore. These results suggested H^+-coupled uphill transport of ceftibuten. In contrast, an inwardly directed Na^+ gradient had no effect on ceftibuten uptake. The valinomycin-induced K^+ diffusion potential (inside positive) significantly stimulated the ceftibuten uptake, suggesting net transfer of the negative charge. In contrast to the *cis*-isomer ceftibuten, the *trans* isomer of ceftibuten is not readily absorbed from the intestine, and its uptake was found not to be affected by a H^+ gradient. Since the lipophilicity of the *trans* isomer is similar to that of ceftibuten, the uptake process appears to be stereoselective. The initial uptake of ceftibuten and its analogue cefaclor was concentration dependent under a H^+ gradient. The apparent K_m value was 0.2 m*M* for ceftibuten and 3.0 m*M* for cefaclor.

KEY WORDS: ceftibuten; proton-coupled uphill transport; rat intestinal brush-border membrane vesicles; stereoselective transport.

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], a new orally active cephalosporin, has a broad antibacterial spectrum (1) and excellent bioavailability in humans (2), dogs, and rats (3). After oral administration of ceftibuten to humans, higher plasma concentrations and a larger AUC value (area under the curve of plasma concentrations) of ceftibuten are expected (2) in comparison with cefaclor (4) and cephalexin (4).

Recent studies with intestinal brush-border membrane vesicles prepared from rats and rabbits demonstrated that the transport of dipeptides (5–7) and amino-β-lactam antibiotics (8–10) was not affected directly by a Na^+ gradient across the membrane, but was markedly enhanced by an inwardly directed H^+ gradient. These transport processes were electrogenic and associated with a net transfer of positive charge across the membrane (5,7,10). Furthermore, Okano *et al.* (10) demonstrated H^+-coupled uphill transport of cephradine (an amino-β-lactam antibiotic) via the dipeptide transport system in rabbit intestinal brush-border membrane vesicles.

There are structural similarities between the dipeptides and the amino-β-lactam antibiotics because they have α-amino and carboxyl groups and exist as a zwitterion at the physiological pH. Ceftibuten, on the other hand, does not have an α-amino group and ionizes as a dianion at physiological pH.

In the present study, to elucidate the transport characteristics of ceftibuten, the effects of a H^+ gradient and the membrane potential on the transport of ceftibuten were examined in rat intestinal brush-border membranes.

MATERIALS AND METHODS

Materials

Ceftibuten, the *trans* isomer of ceftibuten, and moxalactam were synthesized by Shionogi Research Laboratories (Osaka, Japan). Cefaclor, cephalexin, and cephalothin were supplied by Eli Lilly (Indianapolis, Ind). Ceftizoxime (Fujisawa Pharmaceutical Co., Osaka, Japan), cefmenoxime (Takeda Chemical Industries, Osaka, Japan), and cephradine (Sankyo Co., Tokyo) were purchased from the cited companies. Tris, Hepes, and Mes were obtained from Nakarai Chemicals, Ltd. (Kyoto, Japan). Cefadroxil, valinomy-

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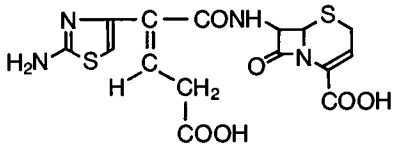

Fig. 1. The chemical structure of ceftibuten.

cin, and DIDS were purchased from Sigma Chemical Co. (St. Louis, Mo.), and FCCP was from Aldrich Chemical Co. (Milwaukee, Wis.). [^{14}C]-D-Glucose (specific radioactivity, 10 mCi/mmol) was from New England Nuclear Corp. (Boston). All other chemicals were of the highest purity available.

Preparation of Brush-Border Membrane Vesicles

Brush-border membrane vesicles were isolated from the small intestine of fasting male Sprague–Dawley rats (200–250 g), according to the calcium precipitation method of Kessler *et al.* (11). All transport studies were performed with membrane vesicles freshly prepared on the day of the experiment.

Protein concentration was measured by the Bio-Rad protein assay kit with bovine γ-globulin as a standard. The activities of alkaline phosphatase (12) and aminopeptidase M (13), the marker enzymes for brush-border membrane, were assayed and the enrichment in the membrane vesicles, compared to that in the starting homogenate, came to 11.3 ± 1.0 (mean ± SE; $N = 5$) and 10.0 ± 0.6 ($N = 5$), respectively. The enrichment factor of (Na^+,K^+)-ATPase (14), the marker enzyme for the basolateral membrane, was 0.39 ± 0.11 ($N = 5$).

To test for the functional integrity of the brush-border membrane vesicles, the Na^+ gradient-dependent D-glucose uptake was examined. Experimental procedures were the same as for ceftibuten described below. A typical overshoot uptake of D-glucose was observed in the presence of an inwardly directed Na^+ gradient (100 mM NaSCN outside, 100 mM KSCN inside) and the peak uptake value was five times higher than that observed at equilibrium (data not shown). These results agree with those of Kessler *et al.* (11) on D-glucose transport by intestinal brush-border membrane vesicles.

Transport Studies

The uptake of ceftibuten by brush-border membrane vesicles was measured by a rapid filtration technique. Most of the uptake studies were performed as follows: membrane vesicles were suspended in 100 m*M* mannitol, 100 m*M* KCl, and 10 m*M* Hepes (pH 7.5) and its protein concentration was prepared to approximately 8–10 mg/ml. Substrate medium was composed of 100 m*M* mannitol, 100 m*M* KCl, 10 m*M* Mes (pH 5.5), and 0.5 m*M* ceftibuten. The reaction was initiated by the addition of 200 μl of substrate medium to 20 μl of membrane suspension at 25°C. After various time intervals, the reaction was stopped by the addition of 1 ml of ice-cold stop solution, containing 100 m*M* mannitol, 100 m*M* KCl, and 10 m*M* Mes (pH 5.5), and immediately filtered through a Millipore filter (DAWP, 0.65 μm, 2.5-cm diameter), followed by washing two times with 3 ml of stop solution. The time required for the washing procedure was less than 10 sec. The ceftibuten trapped on the filter was extracted with 200 μl of distilled water, which was enough to extract more than 90% of ceftibuten trapped on the filter, and measured by high-performance liquid chromatography (HPLC).

FCCP, dissolved in absolute ethanol, was added to the membrane suspension and the final concentrations of FCCP and ethanol were 50 μ*M* and 0.5%, respectively; the control had 0.5% of ethanol alone. DIDS was dissolved in the substrate medium to the final concentrations of 100 μ*M* and 1 m*M*. Valinomycin, dissolved in absolute ethanol, was added to the membrane suspension and the final concentrations of valinomycin and ethanol were 10 μg/mg protein and 0.17%, respectively. Other specific conditions for each experiment are given in the results.

All data shown in the figures represent the mean ± SE of three or more experiments.

Determination of Apparent Partition Coefficients

Apparent partition coefficients of ceftibuten, the *trans* isomer of ceftibuten, and some cephalosporins between 2-methylpropanol and water at pH 5.5 and 7.4 were measured as described by Tsuji *et al.* (15). All procedures were performed at 37°C. The concentrations of cephalosporins in the aqueous phase were determined by HPLC.

Analytical Methods

The concentrations of ceftibuten, the *trans* isomer of ceftibuten, and cefaclor were determined using an LC-6A HPLC (Shimadzu Co., Kyoto, Japan) equipped with an SPD-6A UV detector (Shimadzu Co., Kyoto, Japan) at 262 nm. The chromatograph was equipped with a column with Nucleosil $_{10}C_{18}$ (Chemco Scientific Co., Ltd., Osaka, Japan) for ceftibuten and the *trans* isomer of ceftibuten or Nucleosil $_{7}C_{18}$ for cefaclor. The mobile phase was 8% methanol–100 m*M* ammonium acetate for ceftibuten and the *trans* isomer of ceftibuten and 12% acetonitrile–100 m*M* ammonium acetate for cefaclor. The flow rate was 1.0 ml/min and the injection volume was 100 μl.

For [^{14}C]-D-glucose uptake, radioactivities were measured using a Packard Model 460c liquid scintillation counter (Packard Instruments Corp., Downers Grove, Ill.).

RESULTS

Effect of a Transmembrane Na^+ Gradient on Ceftibuten Uptake

The effect of a Na^+ gradient on ceftibuten uptake was examined at the extravesicular pH of 5.5 and 7.5. Ceftibuten uptake was linear for at least 15 sec at both pH. An inwardly directed Na^+ gradient did not stimulate the initial uptake for 15 sec at either pH (Fig. 2), while the uptake at pH 5.5 increased markedly regardless of a Na^+ gradient. These results clearly suggest that ceftibuten uptake by the brush-border membrane is independent of a Na^+ gradient but dependent on a H^+ gradient. Details for ceftibuten uptake under a H^+ gradient are described below.

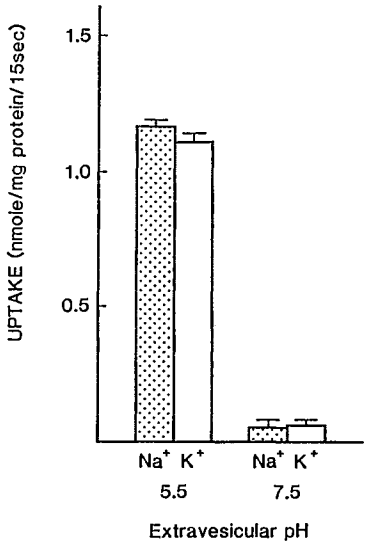

Fig. 2. Effect of a Na^+ gradient on ceftibuten uptake by intestinal brush-border membrane vesicles at two different extravesicular pH values, 7.5 and 5.5. Na^+ and K^+ at the bottoms of the columns indicate the presence and absence of the Na^+ gradient, respectively.

Effect of a Transmembrane H^+ Gradient on Ceftibuten Uptake

The effect of extravesicular pH on ceftibuten uptake was examined. The external pH had a marked effect on the initial uptake of ceftibuten for 15 sec and the most stimulated uptake was observed at an external pH of 5.5 (Fig. 3).

The time course of ceftibuten uptake was examined in the presence and absence of a H^+ gradient. As shown in Fig. 4, an inwardly directed H^+ gradient (pH 7.5 inside, pH 5.5 outside) markedly stimulated the uptake in comparison with the case in which there was no H^+ gradient (pH 5.5 inside, pH 5.5 outside). Furthermore, the ceftibuten uptake for 30 sec was four times greater than that observed at equilibrium (overshoot phenomenon) under a H^+ gradient. In the absence of this gradient, no overshoot was observed. The uptakes of ceftibuten at equilibrium were almost the same irrespective of the presence and absence of a H^+ gradient.

These results strongly suggest that H^+ gradient-dependent uphill transport of ceftibuten occurs in rat intestinal brush-border membranes.

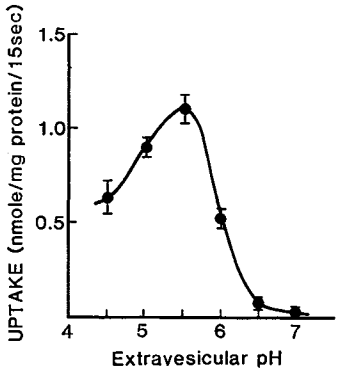

Fig. 3. Effect of extravesicular pH on ceftibuten uptake by intestinal brush-border membrane vesicles. Transport was measured in acetate buffer (pH 4.5–5.0) or Mes buffer (pH 5.5–7.0).

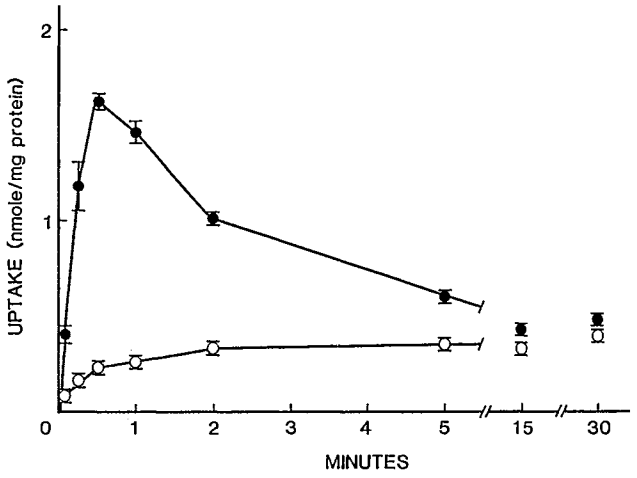

Fig. 4. Effect of an inwardly directed H^+ gradient on ceftibuten uptake by intestinal brush-border membrane vesicles. Membrane vesicles were suspended in either Hepes buffer (pH 7.5) (○) or Mes buffer (pH 5.5) (●) and then transport was studied.

Effects of FCCP and DIDS on Ceftibuten Uptake

The effects of FCCP, a protonophore, and DIDS, an inhibitor of the erythrocyte anion-exchange process, on ceftibuten uptake were examined in the presence of an inwardly directed H^+ gradient. As shown in Fig. 5, the uptake of ceftibuten for 15 sec significantly decreased in the presence of FCCP (50 μM). There appeared to be no effect of DIDS at the low concentration (10 μM) on the uptake of ceftibuten for 15 sec, but a significant decrease in ceftibuten uptake was observed at higher DIDS concentrations (100 μM, 1 mM).

Effect of the Medium Osmolarity on Ceftibuten Uptake

To evaluate the uptake of ceftibuten into an osmotically sensitive space (intravesicular space), the medium osmolarity was varied by the addition of cellobiose. The uptake for 30 min (at equilibrium) decreased with an increase in the medium osmolarity, showing an inversely proportional rela-

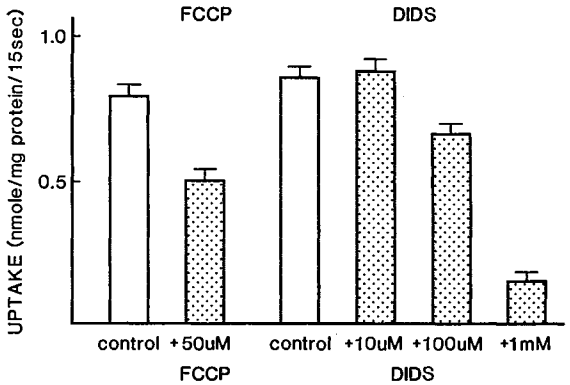

Fig. 5. Effect of FCCP and DIDS on ceftibuten uptake by intestinal brush-border membrane vesicles. Treatment of FCCP or DIDS is described under Materials and Methods. Ceftibuten uptake was measured in the presence of a H^+ gradient.

tionship (Fig. 6). The y intercept, representing the uptake at infinite osmolarity, was almost zero. These results suggest that ceftibuten uptake by brush-border membrane vesicles represents transport into the intravesicular space.

Effect of the Transmembrane Potential on Ceftibuten Uptake

The effect of a K^+ diffusion potential (interior-positive) induced by valinomycin on ceftibuten uptake was studied in the presence of an inwardly directed H^+ gradient (Fig. 7). The membrane vesicles were prepared with 100 mM Na_2SO_4 and the transport medium contained 100 mM K_2SO_4, which resulted in an inwardly directed K^+ gradient. The addition of valinomycin under these conditions generated an interior-positive membrane potential and the uptake of ceftibuten significantly increased at all times measured ($P < 0.01$). These results indicate that ceftibuten transport in intestinal brush-border membrane vesicles is an electrogenic process and results in a net transfer of negative charge.

Uptake Kinetics of Ceftibuten and Cefaclor

The concentration dependency of ceftibuten uptake in the presence of a H^+ gradient was examined. Figure 8 shows a typical plot of the relationship between the uptake for 15 sec and the concentration in the medium of ceftibuten or cefaclor. The initial rate of ceftibuten or cefaclor uptake can be expressed by the following equation:

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

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 rate constant of simple diffusion. Kinetic parameters K_m, V_{max}, and K_{dif} were estimated by NONLIN (16) and their values were 0.17 mM, 4.72 nmol/mg protein/min, and 0.30 nmol/mg protein/min/mM for ceftibuten and 3.0 mM, 4.3 nmol/mg protein/min, and 0.16 nmol/mg protein/min/mM for cefaclor, respectively.

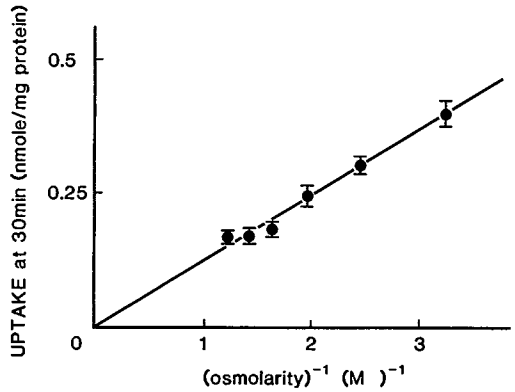

Fig. 6. Effect of medium osmolarity on ceftibuten uptake at equilibrium by intestinal brush-border membrane vesicles. Ceftibuten uptake was measured at 30 min of incubation in the substrate medium containing various concentrations of cellobiose (0–500 mM).

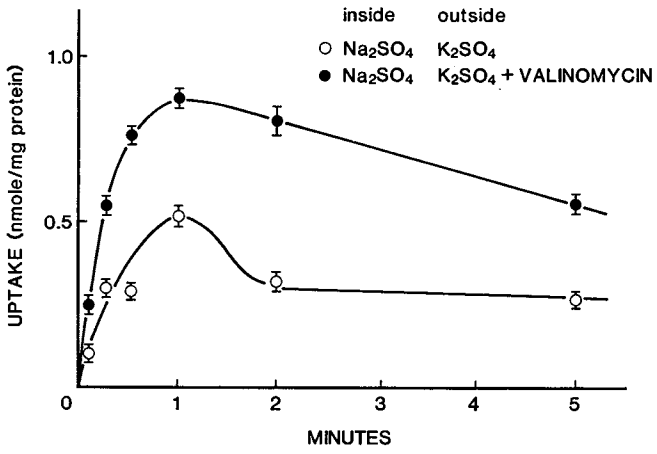

Fig. 7. Effect of membrane potential on ceftibuten uptake by intestinal brush-border membrane vesicles. Membrane vesicles were suspended in Na_2SO_4, mannitol, and Hepes (pH 7.5). The substrate medium was composed of K_2SO_4, mannitol, Mes (pH 5.5), and ceftibuten. Ceftibuten uptake was measured in the presence (●) and absence (○) of valinomycin in membrane suspension.

Uptake of the Geometrical Isomer of Ceftibuten by Brush-Border Membrane Vesicles

Ceftibuten, which has a double bond in its side chain at the 7 position, is the *cis* isomer. When the uptake of the *trans* isomer of ceftibuten was studied in the presence and absence of a H^+ gradient, no effect of this gradient was noted on the uptake and no overshoot was observed (Fig. 9). To determine whether the difference of transport characteristics of these isomers is due to a difference in lipophilicity, their partition coefficients were examined. The values for ceftibuten, the *trans* isomer of ceftibuten, and several other cephalosporins differed markedly (Table I). The lipophilicities of ceftibuten and the *trans* isomer of ceftibuten were similar but lower than those of other cephalosporins, except moxalactam. The increase in the partition coefficient of ceftibuten

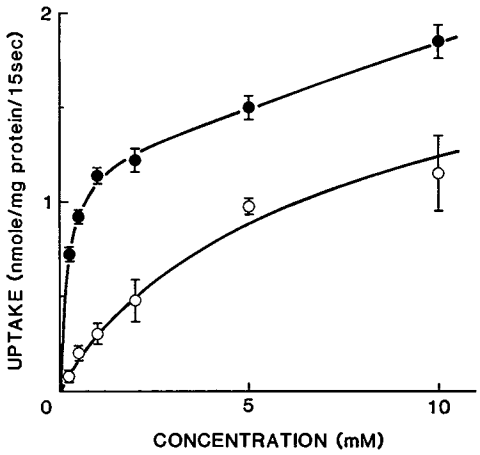

Fig. 8. Concentration-dependent uptake of ceftibuten and cefaclor by intestinal brush-border membrane vesicles. Uptakes of ceftibuten (●) and cefaclor (○) at various concentrations (0.2–10 mM) were measured for the initial 15 sec of incubation in the presence of a H^+ gradient. The curve was generated from Eq. (1) using kinetic parameters estimated by NONLIN.

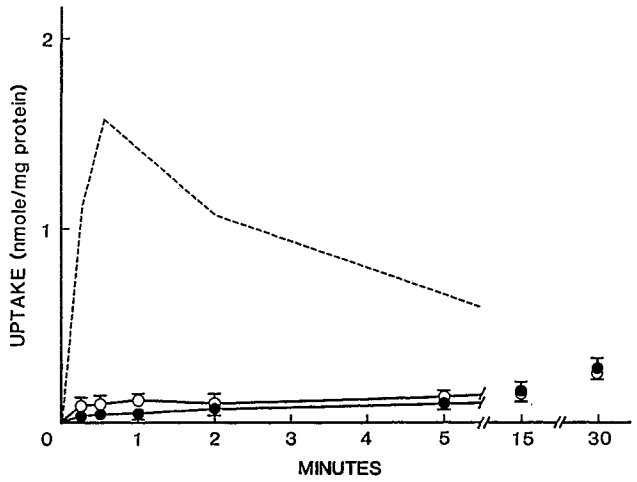

Fig. 9. Transport of the geometrical isomer of ceftibuten by intestinal brush-border membrane vesicles. Uptake of the *trans* isomer of ceftibuten was measured in the presence (●) and absence (○) of a H^+ gradient. The experimental conditions were the same as in the legend to Fig. 4. The dashed line indicates the uptake of ceftibuten in the presence of a H^+ gradient.

with acidic pH may be due to the increase in the nonionized form of ceftibuten. Note that the lipophilicities of ceftibuten and the *trans* isomer of ceftibuten at pH 5.5 were almost the same (Table I). These results suggested that stereoselective uptake of ceftibuten seems to occur in the intestinal brush-border membrane vesicles.

DISCUSSION

The present results show that an inwardly directed H^+ gradient drives uphill transport of ceftibuten in rat intestinal brush-border membrane vesicles. The H^+ gradient transiently produced a fourfold higher ceftibuten concentration than that found at equilibrium (overshoot). This large overshoot phenomenon is remarkable when compared to that of cefaclor (17), cephradine (9,10), or cefixime (18) in intestinal brush-border membrane vesicles from rat or rabbit, and it may result from ceftibuten's high affinity to the transport carrier (K_m = 0.17 m*M*) in comparison with that of cefaclor (K_m = 3.0 m*M*) (in the present study), cephradine (K_m = 9.4 m*M*) (9), and cefixime (K_m = 0.83 m*M*) (18). The H^+ gradient-stimulated uptake of ceftibuten was markedly reduced by the presence of 50 μ*M* FCCP or 1 m*M* DIDS (Fig. 5). These results strongly suggested that the carrier actively cotransports ceftibuten and H^+ (or phenomenologically indistinguishable ceftibuten/OH^- exchange). Na^+ gradients did not stimulate ceftibuten uptake, suggesting that Na^+ gradient is not the driving force of the transport.

Table I. Apparent Partition Coefficients (P_{app}) Between 2-Methylpropanol and Water at 37°C[a]

	pH 5.5	pH 7.4
Moxalactam (*R*)[b]	0.0022 ± 0.0006	0.0016 ± 0.00007
Moxalactam (*S*)[b]	0.0019 ± 0.0003	0.0018 ± 0.0001
Ceftibuten	0.0032 ± 0.0005	0.0011 ± 0.0004
trans-Ceftibuten	0.0034 ± 0.0005	0.0009 ± 0.0005
Cefadroxil	0.016 ± 0.0009	0.031 ± 0.0008
Ceftizoxime	0.033 ± 0.001	0.036 ± 0.006
Cephalexin	0.048 ± 0.0003	0.11 ± 0.005
Cephradine	0.058 ± 0.003	0.13 ± 0.01
Cefmenoxime	0.072 ± 0.002	0.099 ± 0.006
Cephalothin	0.37 ± 0.02	0.41 ± 0.02

[a] Each value represents the mean ± SD of three or more experiments.

[b] *R* and *S* epimers of moxalactam.

Ceftibuten, possessing a double bond in its side chain at the 7 position, is the *cis* isomer. Previous study utilizing the *in situ* loop technique suggested that the *trans* isomer of ceftibuten is not absorbed by rat intestine (19). In the present study, an inwardly directed H^+ gradient had no effect on its uptake (Fig. 9). The lipophilicity of the *trans* isomer of ceftibuten is very similar to that of ceftibuten at pH 5.5 (Table I). Therefore, the carrier may have a high degree of substrate specificity in the transport across the intestinal brush-border membrane.

Ceftibuten uptake by brush-border membrane vesicles was affected by the transmembrane potential. In the presence of a H^+ gradient, ceftibuten uptake was significantly increased by an interior-positive membrane potential generated by valinomycin (an inwardly directed K^+ gradient) (Fig. 7). These results strongly suggest that the uptake is a net transfer of negative charge across the membranes. Ceftibuten has three ionizable groups (i.e., two carboxyl and an aminothiazol groups) with pK_a values of 2.3, 3.2, and 4.5 (20). Therefore, ceftibuten exists predominantly as a dianion at both pH 5.5 and pH 7.5. From these results, it seems that one molecule of ceftibuten is cotransported with one proton across the intestinal brush-border membrane.

The interior of the enterocyte is normally electrically negative relative to the lumen (21), and an acidic microclimate pH exists at the surface of the small intestine, that is, an inwardly directed H^+ gradient exists across the intestinal brush-border membranes (22,23). In the present study, we demonstrated that the transport of ceftibuten was driven by a H^+ gradient and resulted in a net transfer of negative charge. Therefore, it seems that the intestinal absorption of ceftibuten *in vivo* is facilitated by a H^+ gradient and inhibited by the inside-negative electrical potential of the enterocyte. However, excellent absorption of ceftibuten in rat intestine was observed by the *in situ* perfusion and loop technique (19). Consequently, the force of an inwardly directed H^+ gradient may supercede the resistance of the inside negative potential to ceftibuten absorption in intestinal brush-border membranes *in vivo*.

The results of the *in vitro* experiments were compared with those of previous *in vivo* experiments which suggested that ceftibuten is efficiently absorbed in human and animals (2,19), but the *trans* isomer of ceftibuten is not readily absorbed (19). Further studies utilizing *in situ* loop methods suggested that ceftibuten is absorbed by a carrier-mediated process in rat intestine and the absorption is inhibited by dipeptides (19). In the following paper we show that ceftibuten uptake by brush-border membrane vesicles is competitively inhibited by several dipeptides (17). Therefore, the absorption characteristics of ceftibuten in small intestine can be reproduced by the transport in intestinal brush-border membrane vesicles.

REFERENCES

1. T. Yoshida, Y. Hamashima, S. Matsuura, Y. Komatsu, and S. Kuwahara. *26th Intersci. Conf. Antimicrob. Chemother.* (1986), abstr.
2. M. Nakashima, M. Iida, T. Yoshida, T. Kitagawa, T. Oguma, and H. Ishii. *26th Intersci. Conf. Antimicrob. Chemother.* (1986), abstr.
3. K. Hirano, T. Yoshida, T. Matsubara, K. Mizojiri, F. Kobayashi, and S. Kuwahara. *26th Intersci. Conf. Antimicrob. Chemother.* (1986), abstr.
4. T. Kamiki, H. Yamada, and T. Oguma. *Chemotherapy* 27 (S-7):158–174 (1979).
5. V. Ganapathy and F. H. Leibach. *J. Biol. Chem.* 258:14189–14192 (1983).
6. V. Ganapathy and F. H. Leibach. *Am. J. Physiol.* 249:G153–G160 (1985).
7. N. Takuwa, T. Shimada, H. Matsumoto, M. Himukai, and T. Hoshi. *Jpn. J. Physiol.* 35:629–642 (1985).
8. T. Kimura, T. Yamamoto, R. Ishizuka, and H. Sezaki. *Biochem. Pharmacol.* 34:81–84 (1985).
9. T. Okano, K. Inui, M. Takano, and R. Hori. *Biochem. Pharmacol.* 35:1781–1786 (1986).
10. T. Okano, K. Inui, H. Maegawa, M. Takano, and R. Hori. *J. Biol. Chem.* 261:14130–14134 (1986).
11. M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Muller, and G. Semenza. *Biochim. Biophys. Acta* 506:136–154 (1978).
12. O. A. Bessy, O. H. Lowry, and M. J. Brock. *J. Biol. Chem.* 164:321–329 (1946).
13. W. Hasse, A. Schafer, H. Murer, and R. Kinne. *Biochem. J.* 172:57–62 (1978).
14. B. F. Scharschmidt, E. B. Keeffe, N. M. Blankenship, and R. K. Ockner. *J. Lab. Clin. Med.* 93:790–799 (1979).
15. A. Tsuji, O. Kubo, E. Miyamoto, and T. Yamana. *J. Pharm. Sci.* 66:1675–1679 (1977).
16. C. M. Metzler, G. L. Elfring, and A. J. McEwen. *A Users Manual for NONLIN and Associated Programs,* The Upjohn Co., Kalamazoo, Mich., 1974.
17. N. Muranushi, T. Yoshikawa, M. Yoshida, T. Oguma, K. Hirano, and H. Yamada. *Pharm. Res.* 6:308–312 (1989).
18. A. Tsuji, T. Terasaki, I. Tamai, and H. Hirooka. *J. Pharmacol. Exp. Ther.* 241:594–601 (1987).
19. N. Muranushi, T. Yoshikawa, M. Yoshida, T. Oguma, K. Hirano, and H. Yamada. *J. Pharmacobio-Dyn.* 10:s-72 (1987).
20. T. Komeno. Nippon Kagaku Ryoho Gakkai Symposium (1987).
21. S. G. Schultz. In L. R. Johnson (ed.), *Physiology of the Gastrointestinal Tract,* Raven Press, New York, 1981.
22. G. Reckkemmer, M. Wahl, W. Kuschinsky, and W. Engelhardt. *Pflugers Arch.* 407:33–40 (1986).
23. M. L. Lucas. *Gut* 24:734–739 (1983).