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H^+ GRADIENT-DEPENDENT TRANSPORT OF AMINOCEPHALOSPORINS IN RAT INTESTINAL BRUSH-BORDER MEMBRANE VESICLES

ROLE OF DIPEPTIDE TRANSPORT SYSTEM

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Abstract—The transport of cephalosporin antibiotics in brush-border membrane vesicles isolated from rat small intestine has been studied by a rapid filtration technique, demonstrating a carrier-mediated transport system for aminocephalosporins such as cephradine. In agreement with the transport mechanisms of dipeptides, the uptake of cephradine by brush-border membrane vesicles was Na^+-independent and was stimulated in the presence of an inward H^+ gradient ($[H^+]o > [H^+]i$). Cephradine uptake in the presence of an inward H^+ gradient was a saturable process with an apparent K_m of 9.4 mM, and was markedly inhibited by dipeptides but not inhibited by amino acids. The present data suggest that aminocephalosporins can be transported by a common carrier-mediated system with dipeptides in the intestinal brush-border membranes and this process may be driven by an H^+ gradient.

Small intestine and renal proximal tubules absorb various organic and inorganic solutes from the lumen and deliver them to the blood. Recent developments in the isolation of purified brush-border membrane vesicles from epithelial cells of small intestine and kidney have led to a growing number of studies concerning their transport properties such as Na^+ dependence of glucose, amino acid and phosphate transport [1–3]. Thus, transport mechanisms of many nutrients show striking similarities between intestinal and renal brush-border membranes, as well as structure and enzyme pattern.

In the studies for renal membrane transport of organic ions, we have found that aminocephalosporins such as cephalexin and cephradine share a common carrier transport system with dipeptides in renal brush-border membranes, and this system may contribute to tubular reabsorption of aminocephalosporins *in vivo* [4–6]. Using *in situ* perfusion technique, loop technique and *in vitro* everted gut sac technique, several groups have demonstrated that amino-β-lactam antibiotics can be absorbed by a carrier-mediated system in the intestinal mucosa [7–12], and some of them may share a common transport system with dipeptide [8, 12]. Furthermore, it has been reported that brush-border membranes of renal cortex and small intestine possess a dipeptide transport system which transports intact dipeptide by an Na^+-independent and carrier-mediated process [13–16]. These backgrounds prompted the present investigation, which was designed to examine the transport mechanisms of aminocephalosporin antibiotics in the intestinal brush-border membranes, in relation to the carrier-mediated transport system of dipeptides.

MATERIALS AND METHODS

Materials. Cephalexin and cefaclor (Shionogi & Co., Osaka, Japan), cephradine (Sankyo Co., Tokyo, Japan), cefadroxil (Bristol Meyers Co., Tokyo, Japan) and cefazolin (Fujisawa Pharmaceutical Co., Osaka, Japan) were kindly supplied. The structures of cephalosporin antibiotics are illustrated in Fig. 1. Tris and Hepes† were obtained from Nakarai Chemicals, Ltd. (Kyoto, Japan). L-Carnosine (β-alanyl-L-histidine), glycyl-L-proline and glycyl-L-leucine were purchased from Peptide Institute, Inc. (Osaka, Japan) and L-phenylalanylglycine was from Sigma Chemical Co. (St. Louis, MO). All other chemicals used for the experiments were of the highest purity available.

Preparation of brush-border membrane vesicles. Brush-border membrane vesicles were prepared from the small intestine of male Wistar albino rats (200–230 g). The isolation procedure was based on the calcium precipitation method of Will and Hopfer [17] with several modifications. All steps were performed on ice or at 4°. The small intestine was everted, and the mucosa was scraped with glass microscope slides. The scrapings (8 g wet weight from 8 rats) were homogenized with a Universal Homogenizer at full speed for 2 min in 80 ml of 50 mM mannitol, 1 mM Hepes–Tris, pH 7.5 and 0.1 mM PMSF, and then 72 ml of the same buffer was added to make a 5% homogenate. $CaCl_2$ was added to a final concentration of 10 mM, and the homogenate was allowed to stand for 20 min. The homogenate was centrifuged at 6000 *g* for 15 min in

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† Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

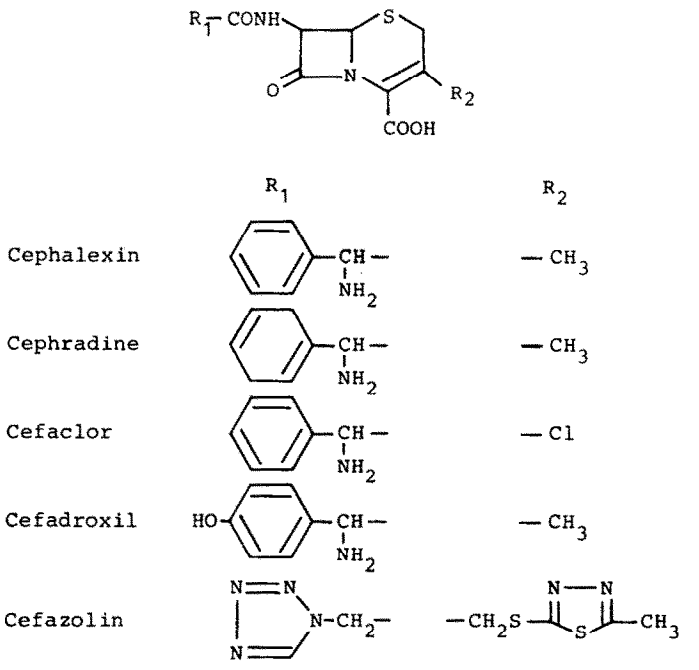

Fig. 1. Structures of cephalosporin antibiotics.

a Hitachi High Speed Refrigerated Centrifuge 20PR-52 (rotor RPR 20–2) (discard pellet 1). The supernate 1 was centrifuged at 21,000 *g* for 30 min (discard supernate 2). The pellet 2 was resuspended in 80 ml of a buffer comprising 100 mM mannitol and 20 mM Hepes–Tris, pH 7.5 (buffer A) plus 0.1 mM PMSF, and homogenized in a glass/Teflon Potter homogenizer with ten strokes at 1000 rpm. $MgCl_2$ was added to a final concentration of 0.1 mM, and the suspension was centrifuged at 6000 *g* for 15 min (discard pellet 3). The supernate 3 was centrifuged at 21,000 *g* for 30 min (discard supernate 4). The pellet 4 was resuspended in 8 ml of a buffer comprising buffer A plus 0.1 mM $MgCl_2$ and 0.1 mM PMSF. The suspension was centrifuged at 3000 *g* for 10 min (discard pellet 5) and the supernate 5 was centrifuged at 21,000 *g* for 30 min (discard supernate 6). The final pellet was resuspended in buffer A plus 0.1 mM PMSF by sucking the suspension ten times through a fine needle (0.4 × 19 mm) with a plastic syringe, and was used for transport studies within 5 hr after preparation.

Transport studies. The uptake of cephalosporins was measured by a rapid filtration technique detailed previously [4, 5], using the incubation media containing 0.1 mM PMSF.

Analytical methods. Cephalosporins were analyzed by a high-pressure liquid chromatograph LC-3A (Shimadzu Co., Kyoto, Japan) with Chemcosorb 5-ODS-H column 15 cm × 4.6 mm (Chemco Scientific Co., Ltd., Osaka, Japan) as described previously [4, 5]. Protein was measured by the method of Bradford [18], using the Bio-Rad Protein Assay Kit, with bovine gamma globulin as a standard. Aminopeptidase (EC 3.4.11.2), $(Na^+ + K^+)$-ATPase (EC 3.6.1.3.), cytochrome *c* oxidase (EC 1.9.3.1.) and lactate dehydrogenase (EC 1.1.1.27) were assayed by the methods as described previously [19]. Sucrase (EC 3.2.1.48) was measured as described by Dahlqvist [20]. β-Glucuronidase (EC 3.2.1.31) was measured as described by Fishman and Bernfeld [21].

RESULTS

In Table 1 are compiled the specific activities and the recoveries of marker enzymes for intestinal brush-border membranes (sucrase, aminopeptidase), basolateral membranes ($(Na^+ + K^+)$-ATPase), lysosomes (β-glucuronidase), mitochondria (cytochrome *c* oxidase) and cytosol (lactate dehydrogenase) during subcellular fractionation of intestinal mucosa. The specific activities of sucrase and aminopeptidase were enriched by a factor of 10 and 11 relative to those in the homogenate, respectively. The final brush-border membrane fraction contains approximately 5% of brush-border marker enzyme activities in the homogenate. In contrast, the contamination by other subcellular fractions was minimal.

To test for functional integrity of the brush-border membrane vesicles, Na^+ gradient-dependent D-glucose uptake was estimated. The membrane vesicles were incubated with 100 mM NaSCN and 0.05 mM [^{3}H]D-glucose, and the uptake of D-glucose was measured as a function of time. A typical overshoot uptake was observed with peak values of 11–14 times the equilibrium (data not shown). This result is similar to other studies with D-glucose transport by intestinal brush-border membrane vesicles.

Figure 2 shows the uptake of various cephalosporin antibiotics (2.5 mM) by brush-border membrane vesicles in the presence of a 100 mM NaCl gradient (outside to inside). The rates of uptake for amino-

Table 1. Specific activities, recoveries and enrichment factors for marker enzymes in brush-border membranes isolated from rat small intestine

	Sucrase		Aminopeptidase		$(Na^+ + K^+)$-ATPase		β-Glucuronidase		Cytochrome *c* oxidase		Lactate dehydrogenase		Protein
	S.A.	%	S.A.	%	S.A.	%	S.A.	%	S.A.	%	S.A.	%	%
Homogenate	95 ± 5	100	48 ± 3	100	41 ± 7	100	1.0 ± 0.1	100	1.02 ± 0.04	100	257 ± 47	100	100
Pellet 1	110 ± 12	73.8 ± 3.4	47 ± 3	63.9 ± 1.3	54 ± 7	87.8 ± 10.6	0.4 ± 0.1	23.6 ± 1.4	1.63 ± 0.08	103 ± 1	37 ± 2	10.4 ± 2.4	64.7 ± 1.5
Supernate 2	5 ± 1	2.2 ± 0.1	16 ± 2	14.0 ± 1.0	4 ± 3	4.3 ± 2.4	1.7 ± 0.3	69.4 ± 4.7	N.D.		568 ± 122	90.5 ± 4.0	41.3 ± 0.1
Pellets 3,5 & supernates 4,6	192 ± 14	3.1 ± 0.5	286 ± 32	9.8 ± 1.4	N.D.		N.D.		N.D.		N.D.		1.5 ± 0.2
Brush-border membranes	957 ± 70	4.9 ± 0.6	529 ± 35	5.0 ± 0.4	6 ± 6	0.1 ± 0.1	N.D.		0.03 ± 0.02	0.01 ± 0.01	5 ± 5	0.01 ± 0.01	0.5 ± 0.1
	(10.1)		(11.0)		(0.15)				(0.03)		(0.02)		

S.A., specific activity (nmol/min per mg protein) (ΔA_{550}/min per mg protein for cytochrome *c* oxidase). % represents the percentage of the enzyme activity found initially in the homogenate. The values in parentheses represent the ratio of the specific activities in brush-border membranes and the homogenate (enrichment factor). N.D., not detectable. Each value represents the mean ± S.E. for 3–4 experiments.

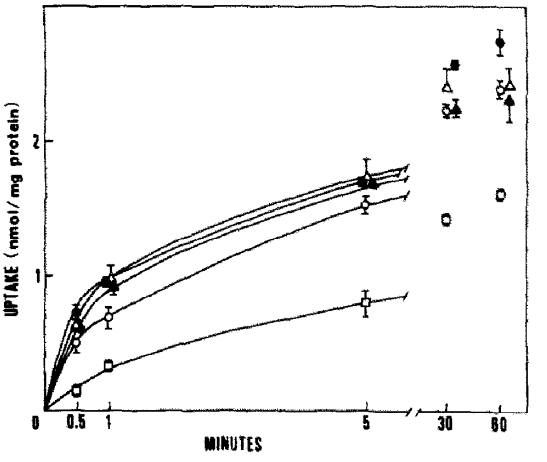

Fig. 2. Uptake of cephalosporin antibiotics by intestinal brush-border membrane vesicles. Membrane vesicles were preincubated at 25° in 100 mM mannitol and 20 mM Hepes–Tris (pH 7.5) for 10 min. The vesicles (20 μl) were incubated at 25° with the substrate mixture (20 μl) comprising 100 mM mannitol, 20 mM Hepes–Tris (pH 7.5), 200 mM NaCl and either 5 mM cephalexin (○), cefaclor (△), cephradine (●), cefadroxil (▲) or cefazolin (□). Final concentration: 100 mM NaCl, 2.5 mM antibiotics. Each point represents the mean ± S.E. of two experiments performed in 1–2 determinations.

cephalosporins such as cephradine, cephalexin, cefaclor and cefadroxil were faster than that for cefazolin, and reached equilibrium at approximately 60 min except for cefazolin. Among the aminocephalosporins, cephradine was chosen as a representative drug in the following studies. Cephradine has two ionizable groups, a carboxyl group with a pKa of 2.6 and an amino group with a pKa of 7.4 [9].

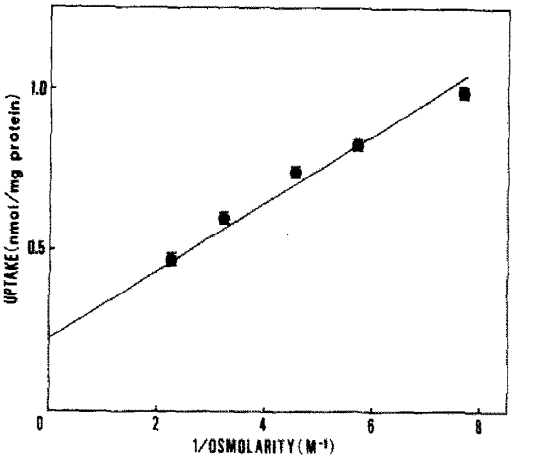

Fig. 3. Effect of osmolarity on the uptake of cephradine by intestinal brush-border membrane vesicles. Membrane vesicles (20 μl, 127 μg protein), suspended in 100 mM mannitol, 20 mM Hepes–Tris (pH 7.5) and 100 mM KCl, were incubated at 25° for 60 min with substrate mixture (20 μl) comprising mannitol, 20 mM Hepes–Tris (pH 7.5), 100 mM KCl and 2 mM cephradine. The osmolarity was varied by addition of mannitol and is shown as the inverse of the medium osmolarity. Each point represents the mean ± S.E. of three determinations.

To ascertain that the uptake of cephalosporins by brush-border membranes represented transport into vesicles rather than membrane binding, cephradine uptake at 60 min was measured when the intravesicular space was decreased by increasing the medium osmolarity with mannitol. As is evident from Fig. 3, there was a linear relationship between cephradine uptake and the reciprocal of the medium osmolarity, suggesting transport into the intravesicular space. Extrapolation of the line to infinite osmolarity, i.e. to zero intravesicular space, indicated that the binding comprised 23% under the conditions normally used.

In order to distinguish whether there is an Na^+-dependent component of the transport, the uptake of cephradine at 1 and 60 min was compared in the presence of an NaCl gradient and a KCl gradient. However, the uptake of cephradine was almost similar in the presence of both ionic gradients (data not shown). The present results are consistent with the uptake characteristics of aminocephalosporins in renal brush-border membranes as reported previously [4, 5].

In renal brush-border membranes, we have demonstrated that aminocephalosporins can be transported via common transport system with dipeptides [5]. Ganapathy and Leibach [22] have recently reported that dipeptide transport in the intestinal and renal brush-border membrane vesicles can be stimulated by an inward H^+ gradient ($[H^+]o > [H^+]i$). Therefore, we have examined the effect of an H^+ gradient on cephradine uptake by intestinal brush-border membrane vesicles. As is evident from Fig. 4, cephradine uptake was stimulated in the presence of an inward H^+ gradient ($[H^+]o > [H^+]i$). The final level of cephradine uptake was approximately similar to that attained in the absence of the gradient or in the presence of the reverse gradient ($[H^+]o < [H^+]i$), suggesting that an equilibrium had been established. Thus, H^+ gradient could be a driving force for cephradine transport in the intestinal brush-border membranes.

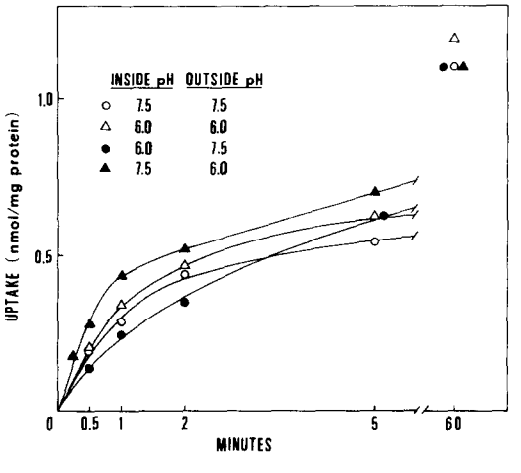

Fig. 4. Effect of H^+ gradient on cephradine uptake by intestinal brush-border membrane vesicles. Membrane vesicles (20 μl, 100 μg protein for pH 7.5, 74 μg protein for pH 6.0), suspended in 100 mM mannitol, 100 mM KCl and either 10 mM Hepes (pH 7.5) (○, ▲) or 10 mM Mes (pH 6.0) (●, △), were incubated with the substrate mixture (200 μl) comprising 100 mM mannitol, 100 mM KCl, 1 mM cephradine and either 10 mM Hepes (pH 7.5) (○, ●) or 10 mM Mes (pH 6.0) (△, ▲). Each point represents the mean value of two determinations.

Table 2. Effect of amino acids (A) and dipeptides (B) on cephradine uptake by intestinal brush-border membrane vesicles

Addition		Cephradine uptake pmol/mg protein per min	%
(A)	Amino acids		
	None	438 ± 7	100
	L-Proline	433 ± 11	99
	L-Phenylalanine	421 ± 27	96
	L-Histidine	419 ± 27	96
	Glycine	410 ± 9	94
(B)	Dipeptides		
	None	450 ± 14	100
	L-Carnosine	338 ± 6*	75
	Glycyl-L-proline	225 ± 4*	50
	Glycyl-L-leucine	176 ± 9*	39
	L-Phenylalanylglycine	174 ± 11*	39

Membrane vesicles (20 μl, 142 μg protein), suspended in 100 mM mannitol, 100 mM Hepes (pH 7.5) and 100 mM KCl, were incubated at 25° for 1 min with substrate mixture (200 μl) comprising 100 mM mannitol, 10 mM Mes (pH 6.0), 100 mM KCl, 1.1 mM cephradine and either 22 mM amino acid or 22 mM dipeptide. Final concentration: 1 mM cephradine and 20 mM amino acid or 20 mM dipeptide. Each value represents the mean ± S.E. of four determinations.

* $P < 0.001$, when compared with control value.

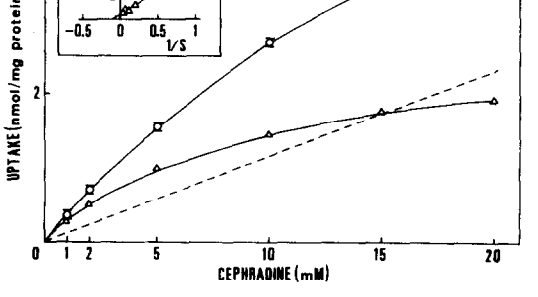

Fig. 5. Concentration dependence of cephradine uptake by intestinal brush-border membrane vesicles. The uptake for 30 sec at concentration between 1 and 20 mM was determined. The vesicles (20 μl, 136 μg protein), suspended in 100 mM mannitol and 10 mM Hepes (pH 7.5) and 100 mM KCl, were incubated at 25° with the substrate mixture (200 μl) comprising 100 mM mannitol, 10 mM Mes (pH 6.0), 100 mM KCl and cephradine. The curves were generated from equation (1) using the fitted kinetic parameters. Solid lines indicate the total uptake (○) and carrier-mediated uptake (△), and dashed line indicates the nonsaturable component (simple diffusion). The inset shows a Lineweaver–Burk plot of cephradine uptake after correction for the nonsaturable component. Each value for total uptake represents the mean ± S.E. of three determinations.

Furthermore, the effects of amino acids and dipeptides (20 mM) on the uptake of cephradine (1 mM) in the presence of an inward H^+ gradient were examined. As shown in Table 2, dipeptides showed a marked inhibitory effect on cephradine uptake by intestinal brush-border membrane vesicles, although the uptake was not inhibited by free amino acids. These results suggest that cephradine and dipeptides could be transported by a common carrier system in the intestinal brush-border membranes as well as renal brush-border membranes.

Figure 5 shows the curve for the concentration dependence of cephradine uptake in the presence of an inward H^+ gradient by intestinal brush-border membrane vesicles. As H^+ gradient-dependent uptake of cephradine appeared to be linear for 30 sec (Fig. 4), the initial rate of cephradine uptake was measured at 30 sec. The relationship between concentration and rate of total uptake approached saturation, but never attained it. An explanation for this phenomenon is that cephradine enters the vesicles by two routes: carrier-mediated process and simple diffusion. Therefore, the initial rate of cephradine uptake can be expressed by the following equation:

$$V = \frac{V_{max}[S]}{K_m + [S]} + K_d[S] \quad (1)$$

where V is the initial uptake rate, [S] is the initial concentration, V_{max} is the maximum uptake rate by carrier-mediated process, K_m is the Michaelis constant, and K_d is the coefficient of simple diffusion. Kinetic parameters were calculated from equation 1 using nonlinear least squares regression analysis [23]. The values of apparent K_m, V_{max} and K_d were 9.4 mM, 5.7 nmol/mg protein/min and 0.2 nmol/mg protein/min/mM, respectively. A Lineweaver–Burk plot of cephradine uptake after correction for the nonsaturable component is shown in Fig. 5 (inset).

DISCUSSION

The present results have demonstrated that aminocephalosporins, zwitterionic drug, can be transported, in part, via dipeptide transport system in the intestinal brush-border membranes: namely (i) the uptake of cephradine was Na^+-independent; (ii) the uptake was stimulated by an inwardly directed H^+ gradient (Fig. 4); (iii) the uptake was inhibited by dipeptides but not by amino acids (Table 2); (iv) the uptake was saturable (Fig. 5).

It is well known that amino-β-lactam antibiotics are efficiently absorbed from the small intestine even though they are ionized at physiological pH and have very low lipid solubilities. In general, present results are comparable to those of several groups which have demonstrated that aminocephalosporins and aminopenicillins are absorbed by a carrier-mediated system in the small intestine [7–12], and some of them may share a common transport system with dipeptide [8, 12]. However, there is apparent discrepancy about the role of Na^+ in the process. The present studies have failed to demonstrate a role of an Na^+ gradient in the transport of aminocephalosporins across intestinal brush-border membranes, as well as renal brush-border membranes [4, 5], although the investigations using intact tissue preparations have shown that the absorption of amino-β-lactam antibiotics is Na^+-dependent [11] and occurs against a concentration gradient [7, 11, 12]. Recently Kimura *et al.* [24] also reported Na^+-independent carrier-mediated transport of cefadroxil in the intestinal brush-border membranes. In addition, the Na^+-independent nature of dipeptide transport in the intestinal and renal brush-border membranes has been shown for a variety of dipeptides [13–16].

Ganapathy *et al.* [22, 25] reported that transport of glycyl-L-proline and glycylsarcosine was stimulated by an inward H^+ gradient ($[H^+]o > [H^+]i$). Furthermore, Takuwa *et al.* [26] demonstrated that overshoot uptake of glycylglycine was observed when an inward H^+ gradient and inside-negative potential difference were imposed simultaneously across brush-border membranes. In the present study, the initial rate of cephradine uptake was also stimulated in the presence of an inward H^+ gradient. The present data, together with the findings of H^+-dependent transport for dipeptides [22, 25, 26], indicate that an inward H^+ gradient may be a driving force for aminocephalosporin transport in the intestinal brush-border membranes.

The luminal pH is acidic compared to intracellular pH, and the brush-border Na^+–H^+ antiport system is largely responsible for the maintenance of this gradient [27]. In the physiological conditions, therefore, it is conceivable that the presence of Na^+ in the lumen stimulates Na^+–H^+ antiport system which results in an increase of an inward H^+ gradient across brush-border membranes ($[H^+]$lumen $> [H^+]$cell) and the condition of acidic luminal pH in turn may accelerate aminocephalosporin transport. Thus, Na^+ dependence of amino-β-lactam antibiotic transport observed in intact tissue preparations [11] might be explained as an indirect phenomenon. On the other hand, the apparent interaction between Na^+ and aminocephalosporins could not be observed in brush-border membrane vesicles because of rapid dissipation of the Na^+ gradient due to the absence of $(Na^+ + K^+)$-ATPase.

In renal brush-border membranes, aminocephalosporins can be transported not only via dipeptide transport system [4, 5] but also via organic cation transport system [6]. As shown in Figs. 2 and 3 of ref. 6, the presence of an outward H^+ gradient ($[H^+]o < [H^+]i$) induced a marked stimulation of aminocephalosporin uptake in agreement with the transport mechanism of organic cation (antiport with H^+), suggesting that the transport activity of organic cation transport system seemed to be greater than that of dipeptide transport system. Thus, the transport mechanisms of aminocephalosporins in renal brush-border membranes are more complicated compared to intestinal brush-border membranes.

Furthermore, it is also necessary to discuss whether the alteration of the extravesicular pH from 7.5 to 6.0 would increase the lipophilicity of cephradine. Concerning the pH-partition behavior of amino-β-lactam antibiotics, Purich *et al.* [28] reported that the anion was the major partitioning species and minimum partitioning occurred in the isoelectric region, suggesting that the zwitterion did not partition to the lipid phase. In the present study,

therefore, it is reasonable to assume that the stimulation of cephradine uptake at the extravesicular pH 6.0 is not due to the increase of its lipophilicity.

In conclusion, we have demonstrated that aminocephalosporins can be transported, in part, by a carrier-mediated system in the intestinal brush-border membranes and that this process is a common carrier transport system with dipeptides, which could be driven by an inward H^+ gradient.

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