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Stereospecific Absorption and Degradation of Cephalexin

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Abstract—Stereospecific absorption and degradation of two stereoisomers about the α-amino group at the 7-position of cephalexin (CEX) have been investigated in the rat intestine. The L-isomer (L-CEX) was not found to be present either in serum or urine after oral administration, but the D-isomer (D-CEX) was well absorbed. In contrast to the saturable uptake of D-CEX ($Kt = 10{\cdot}54 \pm 1{\cdot}73$ mM, pH = 6·0) by the in-vitro everted intestinal sac, no appreciable uptake of L-CEX was observed. However, L-CEX competitively inhibited the uptake of D-CEX by the in-vitro everted intestine and the inhibitory constant (K_i) of L-CEX was determined to be $0{\cdot}67 \pm 0{\cdot}09$ mM. L-CEX was rapidly degraded in-vitro in the intestinal tissue homogenate, serum and urine, while there was no appreciable degradation of D-CEX. Analysis of the major metabolite of L-CEX by high-performance liquid chromatography identified it as 7-aminodeacetoxycephalosporanic acid (7-ADCA). Furthermore, 7-ADCA was detected in serum after oral administration of L-CEX, indicating significant absorption of L-CEX as well as D-CEX. The results obtained suggest that both L- and D-CEX can be absorbed through the intestinal brush-border membrane via the same mechanism, most likely through the dipeptide transport system, and that the affinity of L-CEX to the carrier system is higher than that of D-CEX. However, owing to L-CEX having a higher affinity to the hydrolysing enzymes that are present in the intestinal tissue, serum, and urine, intact L-CEX was immediately hydrolysed after absorption and was not detectable in these samples. These results, demonstrating stereospecific transport and metabolism of CEX, provide strong evidence for a mechanism of carrier-mediated uptake in the absorption of CEX.

Some β-lactam antibiotics, particularly the derivatives of amino-β-lactam antibiotics with an α-amino group in the side chain (the 6-position in penicillins and the 7-position in cephalosporins), have been confirmed to be absorbed mainly via the dipeptide transport system existing in the intestinal brush-border membrane (Addison et al 1975; Kimura et al 1978; Nakashima et al 1984a, b; Okano et al 1986; Tsuji et al 1981, 1987). In the side chains of amino-β-lactam antibiotics there is an asymmetric carbon, and all of these antibiotics commercially available are composed of the D-isomer. Since the endogenous amino acids in mammals are L-isomers, the β-lactam derivatives containing L-amino acids are expected to be more readily transported by the dipeptide carrier system than the D-isomers. Indeed, a higher affinity of dipeptides composed of the L-amino acids to the dipeptide carrier system has been demonstrated by several investigators (Asatoor et al 1973; Addison et al 1975; Das & Radhakrishnan 1975; Ganapathy & Leibach 1982a). However, no studies to date, except for a study by Sullivan et al (1969), have been reported concerning the differences in intestinal transport characteristics of the stereoisomers of β-lactam antibiotics following their oral administration. Sullivan et al (1969) found no intact form of the ^{14}C-labelled L-isomer of cephaloglycin (CEG), an amino-β-lactam antibiotic, in serum or urine following its oral administration to rats, although about half of the radioactivity was recovered in urine. On the contrary, after the administration of the D-isomer, they reported the finding of deacetylcephaloglycin as a major metabolite, as well as CEG itself in the urine. This suggests the occurrence of stereoselective inactivation or absorption of CEG in the rat. However, they did not study further details of the intestinal absorption characteristics of stereoisomers of CEG.

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The purpose of this study was to investigate the stereospecificities of the intestinal dipeptide transport system and metabolic enzymes to β-lactam antibiotics using one of the optically active β-lactam derivatives, cephalexin (CEX) as a model compound; thereby, clarifying a conflicting report (Halpin et al 1980), in which D-CEX was taken up by everted intestine via a simple diffusion, to previous investigations (Nakashima et al 1984a, b) regarding the participation of a carrier-mediated transport mechanism in the absorption of D-CEX.

Materials and Methods

Animals

Male Wistar rats, 200–240 g, (Sankyo Laboratory Co., Toyama, Japan) were fasted for 20 h before the experiment, with water given freely.

Chemicals

The D-isomer of cephalexin (D-CEX) was kindly supplied by Shionogi & Co., Osaka, Japan and the L-isomer (L-CEX, custom-made) was obtained from Nippon Bulk Yakuhin Co., Ltd., Osaka, Japan. [^{14}C]Inulin (5 mCi $mmol^{-1}$) was purchased from Amersham International Ltd, Bucks, UK. All other chemicals were of reagent grade and used without further purification.

Absorption studies

In experiments involving oral administration, rats were anaesthetized with pentobarbitone and cannulae were inserted into the femoral artery and urinary bladder to collect blood and urine samples. A 50 mg kg^{-1} dose of L- or

D-CEX dissolved in water was administered (about 1 mL) via a gastric tube. For blood sampling, the femoral cannula was heparinized.

Experimental methods and measurements of influx in the in-vitro everted intestinal sacs were identical to those described previously (Nakashima et al 1984b). The quantity of CEX taken up was corrected for the volume of extracellular fluid (inulin space) adhering to the mucosal surface. The inulin space was determined using [^{14}C]inulin as described by Nakashima et al (1984b). The incubation medium used was a Krebs-Ringer Tris buffer solution (pH = 6·0) containing (mM) NaCl 118, Tris 25, KCl 4·7, $CaCl_2$ 2·5, $MgSO_4$ 1·2 and KH_2PO_4 1·2. The rate of uptake was determined for the first 4 min of incubation after a 5 min pre-incubation with oxygen at 37 °C.

Degradation studies

Blood and urine samples were collected from ether-anaesthetized rats through cannulae inserted into the carotid artery and urinary bladder. Serum was obtained by centrifuging the blood samples at 2000 *g* for 20 min. L- or D-CEX dissolved in distilled water was mixed with the serum or urine samples in polyethylene tubes to a final concentration of 100 μM, and the reaction was started at 37°C. Intestinal tissue was homogenized (Ulta Turrax, Ika-Werk, Janke & Kunkel) in Krebs-Ringer Tris buffer solution to yield a 40% (w/v wet) homogenate. Equal volumes of the samples containing L- or D-CEX were added to the intestinal homogenate, and then the reaction was started by incubating at 37°C. In the experiments with respect to the heat-denatured samples, serum and intestinal homogenate were boiled for 10 min before the initiation of the reaction.

Analytical procedures

In the oral administration and in-vitro degradation studies, an aliquot of the tissue homogenate, serum or urine was deproteinized by mixing well with an equal volume of methanol and then centrifuged with an Eppendorf centrifuge (Eppendorf, 5412) for 5 min. The resultant supernatant was filtered through a Millipore filter (0·45 μm). In the experiments of uptake by the in-vitro intestinal everted sac, the intestinal tissue was cut into small pieces at the end of the uptake period, and homogenized in ice-cold 1/15 M phosphate buffer (pH = 7·4) to yield a 20% (w/v wet weight) homogenate. After a 15 min centrifugation, the resultant supernatant was deproteinized as described above. Quantities of L- and D-CEX in the deproteinized samples were determined by HPLC. The HPLC system (model BIP-I, Japan Spectroscopic Co., Tokyo, Japan) was equipped with a UV detector (model UVIDEC-100 V, Japan Spectroscopic Co.) and a recorder-integrator (model Chromatopak CR-3A, Shimadzu Co., Kyoto, Japan). The analytical column used was a reversed phase TSK-gel ODS-80 TM (4·6 mm × 15 cm, Toyosoda Industry Co., Tokyo). In addition, a C_{18}/Corasil guard column (Waters Associate, Milford, Mass.) was used between the analytical column and injector. The mobile phase was 20% methanol–80% water containing 10 mM ammonium acetate for D-CEX, and 10% methanol-90% phosphate buffer (10 mM, pH 3·0) containing 10 mM ammonium acetate and 10 mM sodium pentane sulphonic acid for L-CEX. Flow rate of the mobile phase was 1·0 mL min^{-1} and the effluent was monitored at 260 nm. The calibration curves were generated after deproteinization with methanol using a blank tissue homogenate, serum, or urine samples containing known quantities of L- or D-CEX. The metabolite of L-CEX was detected with a Develosil, reversed phase column (4·6 × 150 mm, Nomura Chemical Co., Seto, Japan). The mobile phase was 8% acetonitrile–92% water containing 10 mM ammonium acetate and 10 mM tetra-*N*-butylammonium bromide.

The [^{14}C]inulin radioactivities were measured by liquid scintillation counting of vials containing 10 mL of toluene-based scintillation fluid (500 mL toluene, 500 mL Triton X-100, 6·0 g 2,5-diphenyloxazole, 75 mg 1,4-bis-(2-(5-phenyloxazolyl))benzene). Precisely weighed intestinal samples were oxidized with a sample oxidizer (model ASC-113, Aloka Co., Tokyo) to $^{14}CO_2$, and radioactivity was determined by a liquid scintillation counter (model LSC-700, Aloka Co.).

Data analysis

The kinetic parameters for the uptake of D-CEX were obtained by solving the following equation by non-linear least-squares method using a NONLIN 74 computer program (Metzler et al 1974):

$$J = JmaxC/(Kt + C) + kdC \quad (1)$$

where J and C are the observed uptake rate and the concentration of the substrate in the medium, respectively; and Jmax, Kt, and kd represent the maximum uptake rate, the Michaelis constant and the apparent first-order rate constant, respectively.

Results and Discussion

Time courses for the serum concentrations and cumulative urinary recoveries of L- and D-CEX after oral administration (50 mg kg^{-1}) are shown in Fig. 1a, b. The data obtained for serum concentration and urinary recovery of D-CEX correlated well with previous results (Yoshida et al 1979), indicating that D-CEX was well absorbed and then excreted in urine. On the other hand, L-CEX could not be detected in either serum or urine by the analytical procedures employed in this study at the detection limit of L-CEX of about 1 μg mL^{-1}. It is interesting that the conflicting experimental results were obtained between D- and L-CEX, which suggesting the different disposition of L-isomer from the D-isomer in the body.

Fig. 2 shows the uptake of L- and D-CEX by in-vitro everted intestinal sacs as dependent on concentrations. Uptake was measured at a pH of 6·0, where CEX exists predominantly as a zwitterion, as indicated by the dissociation constants, i.e., $pK_a = 2·64$ for the carboxyl group and $pK_a = 6·96$ for the amino group (Tsuji et al 1981). D-CEX concentration-dependent uptake kinetics were observed to be identical to those reported previously at a pH of 7·0 (Nakashima et al 1984b). The kinetic constants obtained by non-linear least-squares analysis in accordance with equation 1 were: Kt and Jmax (saturable uptake component) = 10·54 ± 1·73 mM and 538·2 ± 69·0 nmol min^{-1} g^{-1} of wet tissue, respectively. Kd (non-saturable uptake component) = 20·6 ± 1·11 nmol min^{-1} g^{-1} of wet tissue mM^{-1}. The

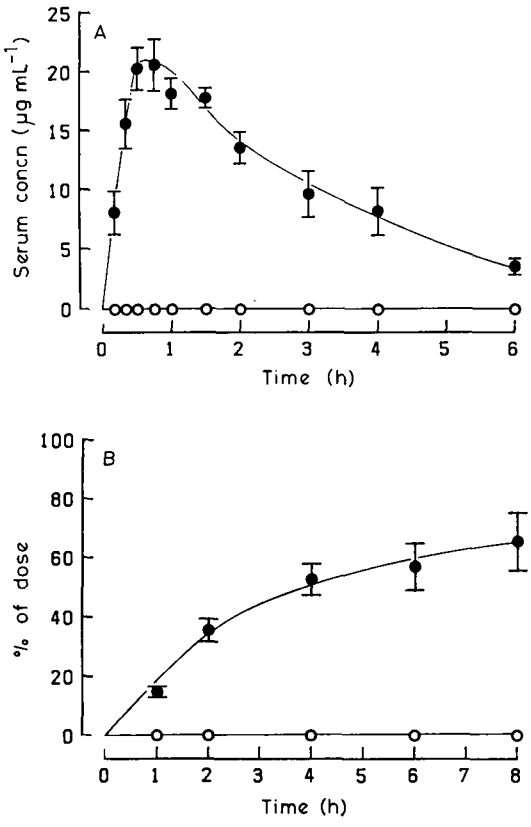

FIG. 1. Time courses for (A) the serum concentration and (B) the percent recovery in urine of D- (●) and L-CEX (○) after a 50 mg kg^{-1} oral administration. Each point represents the mean ± s.e.m. of at least three determinations.

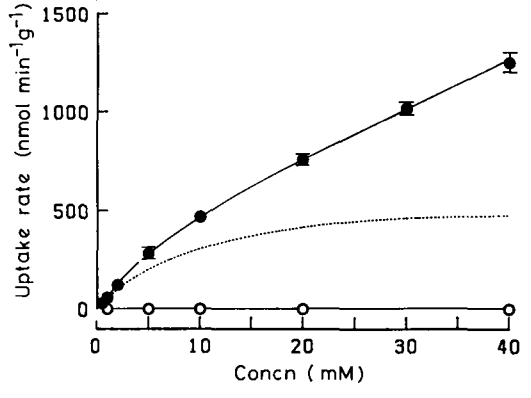

FIG. 2. Concentration dependencies for the uptake of D- (●) and L-CEX (○) by isolated everted intestine of rats. The solid and dotted lines were generated from equation 1, using the NONLIN fitted parameters, indicating total and mediated uptakes of D-CEX, respectively. Each point is the mean ± s.e.m. of at least three determinations.

clearance of uptake (Jmax/Kt) for D-CEX increased at pH 6·0 (51·1 nmol min^{-1} g^{-1} of wet tissue mM^{-1}) compared with a value of 41·8 at pH 7 obtained by Nakashima et al (1984b). This may imply that the H^+-gradient is a driving force in the uptake of this antibiotic via the dipeptide carrier system (Okano et al 1986). Conversely, the uptake of L-CEX was not

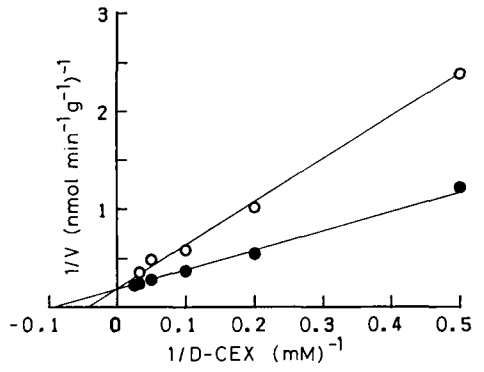

FIG. 3. Lineweaver-Burk plots of uptake of D-CEX in the absence (●) and presence (○) of L-CEX (1 mM). Each value is corrected for non-mediated uptake and represents the mean of at least three determinations.

detectable in the intestinal tissue at the detection limit of about 10 nmol g^{-1} of wet tissue after correction of the tissue samples for the extracellular mucosal space using [^{14}C]inulin.

Lineweaver-Burk plots of the saturable uptake of D-CEX by everted sacs after correction for the nonsaturable component in the presence and absence of 1 mM L-CEX are shown in Fig. 3. L-CEX clearly demonstrated competitive inhibitory effects on the uptake of the D-isomer, and the inhibitory constant of L-CEX as calculated by non-linear least-squares analysis was 0·67 ± 0·09 mM. Because the value of K_i obtained for L-CEX is equal to the Michaelis constant for the uptake of L-CEX in the case of competitive inhibition, it can be said that L-CEX has a higher affinity for the carrier system than has D-CEX. Since dipeptides composed of D-amino acid residues are thought to have a poor affinity to the dipeptide carrier system (Asatoor et al 1973; Addison et al 1975; Das & Radhakrishnan 1975; Ganapathy & Leibach 1982a), it is safe to say that L-CEX has a higher affinity for the binding site on the dipeptide carrier system than has D-CEX, though no significant absorption of L-CEX was observed in either the in-vivo or in-vitro experimental systems.

Two mechanisms are proposed as possible explanations for the above results. The first is that L-CEX specifically binds to a carrier-mediated transport system, but it cannot be translocated by this system. The second is that L-CEX is absorbed through the carrier system, but the antibiotic is rapidly degraded by the hydrolysing enzymes present at the mucosal surface and/or in enterocytes. However; if the specific binding of L-CEX to a carrier system occurs without hydrolysis, a greater quantity of L-CEX is likely to be detected in the in-vitro everted sacs. But this is not what was observed to occur in the present studies. To test the second hypothesis, therefore, the hydrolysing activities of the intestinal tissue, serum, and urine were studied. The time courses for the degradation of L-CEX in the buffer solution (pH 6·0), serum, urine, and intestinal mucosal homogenate together with those in the heat-denatured serum and intestinal homogenate are shown in Fig. 4. These degradations are compared with those of D-CEX. In Fig. 4, the ordinate represents the logarithmic values of the concentrations of L- and D-CEX in the reaction solutions when the initial concentration of CEX was 100 μM. L-CEX was rapidly degraded in the mucosal homogenate, followed by serum

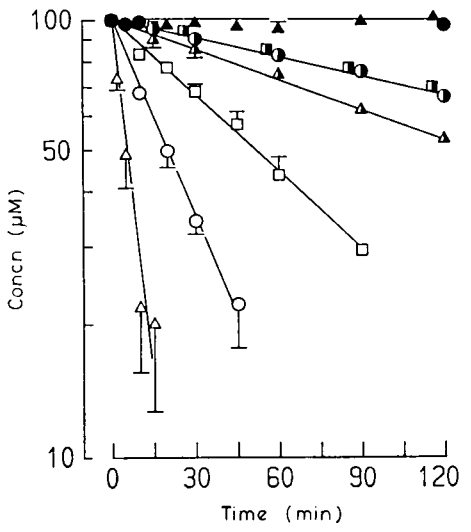

FIG. 4. Degradation of D- (closed symbols) and L-CEX (open symbols) by intestinal tissue homogenate (▲, △, ▲), serum (●, ○, ◑), and urine (□). Half closed symbols represent the results obtained in serum (◑) and intestinal homogenate (▲) boiled for 10 min and in the buffer solution of pH 6·0 (■). The initial concentrations of D- and L-CEX were 0·1 mM, and reacted at 37°C. Each point represents mean ± s.e.m. of at least three determinations.

and urine (calculated half-lives of 5, 20 and 51 min, respectively), while the rates in the heat-denatured biological samples were slow and comparable with that observed in the buffer solution. Interestingly, no appreciable decrease in the concentration of D-CEX was observed in any of the samples. These results suggest that only L-CEX is degraded enzymatically in the tissues and body fluids. Therefore, the second mechanism proposed is more likely and can be said that the L-isomer is subjected to degradation by hydrolysing enzymes with a high-affinity and high capacity, while the D-isomer is resistant to the enzymes. The lack of L-CEX in-vivo following its oral administration (Figs 1, 2) is believed to be due to the degradation of the isomer during the transport process at the intestinal brush-border membrane, in enterocytes, and/or in serum. According to the model proposed by Ganapathy & Leibach (1982b) for dipeptide transport and hydrolysis at the intestinal brush-border, there are two functional sites on the carrier system; one is a translocating site and the other is a hydrolysing site. Only substrates with sufficient affinity to the hydrolysing site are degraded. Applying this model to the transport and degradation of CEX, it is assumed that L-CEX has a higher affinity for the hydrolysing site on the carrier system than the D-isomer and that only the L-isomer is easily degraded at this site during the translocation. Preliminary studies indicate that L-CEX was rapidly degraded when incubated with purified intestinal brush-border membrane vesicles (data not shown). Therefore, most of the L-CEX appears to be hydrolysed at the brush-border membrane rather than in enterocytes. Since a high peptidase activity in the intestinal brush-border membrane is well-known, the results obtained in this study are reasonable. Intact L-isomer passing through the brush-border membrane is also exposed to hydrolysing enzymes in the enterocytes, in the basolateral membrane, in serum and urine. The instability of L-CEX in the presence of such hydrolytic enzymes, therefore, precludes its detection as the intact L-isomer in serum or urine following oral administration.

Many studies have been conducted concerning the action of hydrolysing enzymes on dipeptides at the intestinal brush-border membrane. The activities of the enzymes have been reported to be highly dependent on pH (Wojnarowska & Gray 1975; Burston et al 1982). L-CEX, however, was observed to undergo a pH-independent degradation in a pH range of 5–7 (data not shown). Furthermore, degradation of L-CEX (100 μM, 10 min reaction) observed in the intestinal tissue homogenate at a pH of 6·0 was reduced significantly in the presence of D-CEX at the concentrations of 17 mM to 77·2 ± 2·0% (n = 4, $P < 0.05$). However, neither glycyl-L-proline (20 mM), unstable dipeptide (Ganapathy et al 1981), nor alanine-β-naphthylamide (4 mM), an effective inhibitor of amino-oligopeptidase at the intestinal surface membrane (Kania et al 1977), reduced degradation of L-CEX. These results suggest that the hydrolysing enzymes that act on L-CEX have characteristics different from those of the peptide hydrolases observed previously.

Identification of the degradation products of L-CEX was attempted using HPLC. Possible sites of the L-CEX hydrolysis are the peptide bonds in the side chain at the 7-position and in the β-lactam ring. Sullivan et al (1969) investigated the metabolism of the L-isomer of cephaloglycin (L-CEG) and identified L-2-phenyl-glycine as a metabolite. Fig. 5 shows

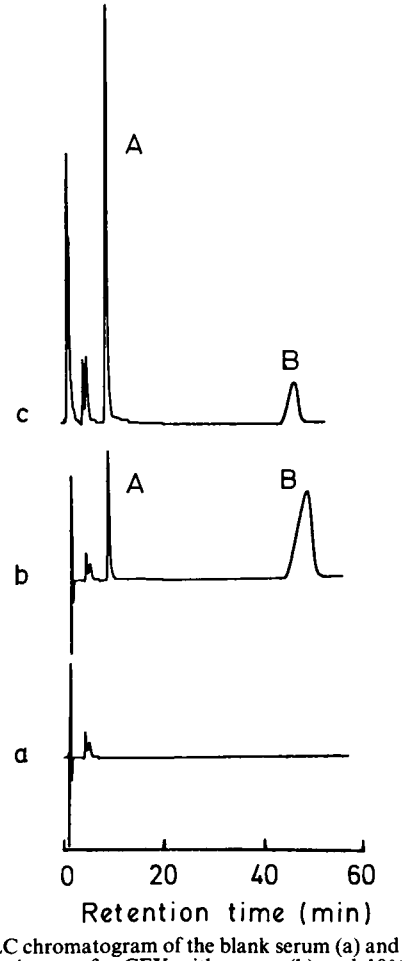

FIG. 5. HPLC chromatogram of the blank serum (a) and the 10 min incubation mixture of L-CEX with serum (b) and 10% intestinal tissue homogenate (c) monitored at 260 nm. Peaks (A) and (B) represent 7-ADCA and L-CEX, respectively.

the HPLC chromatograms of the blank serum (a), L-CEX reacted for 10 min with serum (b) and with intestinal mucosal homogenate (c). By comparison with authentic samples, peaks A and B were identified as 7-aminodeacetoxycephalosporanic acid (7-ADCA) and the intact L-CEX, respectively. The susceptable site on the L-CEX molecule for action by hydrolysing enzymes was suggested to be the same as that observed for L-CEG. However, it is uncertain as to whether the cleavage of the peptide bond of the side chain is an absolute pathway of hydrolysis of L-CEX. Using this analytical method, 7-ADCA was detected in serum after oral administration of L-CEX (50 mg kg^{-1}) to rats and in the intestinal tissue after the uptake by in-vitro everted intestinal sac (data not shown). In another experiment using in-vitro intestinal everted sac, 7-ADCA (2 mM) had no significant inhibitory effect on the uptake of D-CEX (1 mM), though a reduced uptake of D-CEX ($81{\cdot}4 \pm 8{\cdot}5\%$ of control) was observed at the higher concentration of 7-ADCA (10 mM). In contrast, L-CEX significantly reduced the uptake of D-CEX in the lower concentration (2 mM) to $62{\cdot}0 \pm 3{\cdot}1\%$ of control, suggesting a lower affinity of 7-ADCA to the carrier system compared with L-CEX. Therefore, the possibility that 7-ADCA itself is absorbed after the degradation of L-CEX in the mucosal surface via the dipeptide carrier system is unlikely. These results provide evidence that L-CEX itself is transported from the luminal side into the cells via the dipeptide carrier system with a higher affinity than D-CEX and that it is easily hydrolysed in the process of the membrane translocation.

In summary, L-CEX has a higher affinity for the dipeptide carrier system than the D-isomer. However, it is impossible to detect intact L-CEX in the serum or urine following oral administration, owing to the presence of hydrolysing enzymes existing at the intestinal brush-border membrane, in the enterocytes, serum and urine. L-CEX is rapidly degraded through cleavage of the peptide bond in the side chain at the 7-position. Although there has been a conflicting report concerning the participation of a carrier-mediated transport system in the absorption of D-CEX (Halpin et al 1980), the stereospecificity of the transport of CEX confirmed in this study, along with previous observations (Addison et al 1975; Yamashita et al 1984; Nakashima et al 1984 b; Okano et al 1986), strongly suggests that CEX is transported via a carrier system existing in the intestinal brush-border membrane.

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