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# Influence of amino acid side-chain modification on the uptake system for β-lactam antibiotics and dipeptides from rabbit small intestine

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**The influence of chemical modification of functional amino acid side-chains in proteins on the $H^+$-dependent uptake system for orally active α-amino-β-lactam antibiotics and small peptides was investigated in brush-border membrane vesicles from rabbit small intestine. Neither a modification of cysteine residues by $HgCl_2$, NEM, DTNB or PHMB and of vicinal thiol groups by PAO nor a modification of disulfide bonds by DTT showed any inhibition on the uptake of cephalexin, a substrate of the intestinal peptide transporter. In contrast, the $Na^+$-dependent uptake systems for D-glucose and L-alanine were greatly inhibited by the thiol-modifying agents. With reagents for hydroxyl groups, carboxyl groups or arginine the transport activity for β-lactam antibiotics also remained unchanged, whereas the uptake of D-glucose and L-alanine was inhibited by the carboxyl specific reagent DCCD. A modification of tyrosine residues with *N*-acetylimidazole inhibited the peptide transport system and did not affect the uptake systems for D-glucose and L-alanine. The involvement of histidine residues in the transport of orally active α-amino-β-lactam antibiotics and small peptides (Kramer, W. et al. (1988) Biochim. Biophys. Acta 943, 288–296) was further substantiated by photoaffinity labeling studies using a new photoreactive derivative of the orally active cephalosporin cephalexin, 3-[*phenyl*-4-$^3$H]azidocephalexin, which still carries the α-amino group being essential for oral activity. 3-Azidocephalexin competitively inhibited the uptake of cephalexin into brush-border membrane vesicles. The photoaffinity labeling of the 127 kDa binding protein for β-lactam antibiotics with this photoprobe was decreased by the presence of cephalexin, benzylpenicillin or dipeptides. A modification of histidine residues in brush-border membrane vesicles with DEP led to a decreased labeling of the putative peptide transporter of $M_r$ 127000 compared to controls. This indicates a decrease in the affinity of the peptide transporter for α-amino-β-lactam antibiotics by modification of histidine residues. The data presented demonstrate an involvement of tyrosine and histidine residues in the transport of orally active α-amino-β-lactam antibiotics across the enterocyte brush-border membrane.**

## Introduction

The uptake of small peptides in the small intestine and the kidney occurs by saturable transport systems which are stimulated by an inwardly directed $H^+$-gradient ($pH_{out}$ less than $pH_{in}$) [1–6]. The peptide/proton cotransport system is a ternary active transport system; the pH-gradient is generated by a combined action of a $Na^+/H^+$-exchange system in the brush-border membrane and an $Na^+/K^+$-ATPase in the basolateral membrane. The intestinal absorption of orally active α-amino-β-lactam antibiotics is also stimulated by an inwardly directed $H^+$-gradient [7,8] and kinetic studies provided evidence that α-amino-β-lactam antibiotics are taken up by the intestinal uptake system for di- and tripeptides [7–12]. With brush-border membrane vesicles from the small intestine and also from the kidney it was proven by photoaffinity labeling experiments with photoreactive derivatives of penicillins, cephalosporins and dipeptides that peptides and β-lactam antibiotics are

Abbreviations: DCCD, dicyclohexylcarbodiimide; DEP, diethylpyrocarbonate; DFP, diisopropyl fluorophosphate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; NBD-chloride, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; PAO, phenylarsine oxide; PGO, phenylglyoxal; PHMB, 4-hydroxymercuribenzoate; PITC, phenylisothiocyanate; PMSF, phenylmethylsulfonyl fluoride; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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transported by identical transport systems [8,12–15]. With all photoprobes an integral membrane protein of molecular weight 127 000 was identified as a component of the intestinal peptide transport system in the brush-border membrane of enterocytes in the small intestine of rabbit, rat and pig [8,12–14].

Various $H^+$-dependent solute transport systems are found in microorganisms [16–20]. Functional amino acid side-chains of these proteins are involved in the transport processes. Especially thiol- and histidine residues are described to play an essential role in transport processes [18,20–30]. Histidine and thiol groups have been shown to be essential for the activity of the dipeptide/proton cotransport system in rabbit renal brush-border membrane vesicles [29,30]. In the transport system responsible for the resorption of β-lactam antibiotics and small peptides from the small intestine a histidine residue is involved in the transport process of carrier-bound substrate across the intestinal brush-border membrane [31,32]. In order to investigate the molecular interactions necessary for binding and transport of a substrate to the intestinal peptide transport system, we investigated in the present study the influence of chemical modification of different amino acid side-chains on the transport activity of the intestinal uptake system for small peptides and β-lactam antibiotics.

## Materials and Methods

*Materials.* [*phenyl*-4-(n)-$^3$H]Benzylpenicillin (specific radioactivity 18–31 Ci/mmol) obtained from Amersham (Amersham Buchler GmbH, Braunschweig, F.R.G.). D-[U-$^{14}$C]Glucose (specific radioactivity 258.5 mCi/mmol) and L-[3-$^3$H]alanine (specific radioactivity 84 Ci/mmol) were obtained from Du Pont de Nemours (NEN Division, Dreieich, F.R.G.). *N*-(4-Azido[3,5-$^3$H]benzoyl)-cephalexin (specific radioactivity 50.1 Ci/mmol) was synthesized as described [8]. 3-[*phenyl*-4-$^3$H]Azido-cephalexin (specific radioactivity 1.5 Ci/mmol) was synthesized at Hoechst Aktiengesellschaft. Diethylpyrocarbonate (DEP), diisopropylfluorophosphate (DFP) on Kieselgur (5%), dicyclohexylcarbodiimide (DCCD) pentachlorophenol complex, dithiothreitol (DTT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide (NEM), phenazine methosulfate, phenylisothiocyanate (PITC), 4-hydroxymercuribenzoate (PHMB), phenylmethylsulfonyl fluoride (PMSF) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were from Serva (Heidelberg, F.R.G.). Cephalexin, *N*-acetylimidazole, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-chloride), phenylarsine oxide (PAO) and phenylglyoxal (PGO) were bought at Sigma (München, F.R.G.). Mercury dichloride was obtained from Riedel de Haen (Hannover, F.R.G.). Cellulose nitrate filters (type HAWP 0.45 μm, 25 mm diameter) for the transport studies were from Millipore (Eschborn, F.R.G.). Solvents for HPLC were from Merck (Darmstadt, F.R.G.). Acrylamide, *N,N′*-methylenebisacrylamide, Serva Blue R 250 and further chemicals for electrophoresis were obtained from Serva (Heidelberg, F.R.G.). Tissue solubilizer Biolute S and scintillators Quickzint 361 and 501 were obtained from Zinnser Analytic (Frankfurt, F.R.G.). All other substances were from commercial sources and of analytical grade.

*Animals.* White rabbits (3–3.5 kg body weight) (Tierzucht Kastengrund, Hoechst Aktiengesellschaft, Frankfurt am Main, F.R.G.) were maintained on standard diets and tap water ad libitum.

*Preparation of brush-border membrane vesicles from rabbit small intestine.* Brush-border membrane vesicles from rabbit small intestine were prepared by the $Mg^{2+}$-precipitation method as described previously [8,32,33]. The final pellet of the vesicle preparation was suspended in the desired volume of 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol using a No. 27 gauge needle. The enrichment of the specific activities of the brush-border marker enzymes leucine aminopeptidase (EC 3.4.11.2) and γ-glutamyltransferase (EC 2.3.2.2) were $26 \pm 5\times$ and $23 \pm 4\times$, respectively. Protein was determined according to Bradford using the Bio-Rad kit (Bio-Rad München, F.R.G.). The marker enzymes were determined using the Merckotest kits 3359 and 3394 (Merck, Darmstadt, F.R.G.). The quality of the vesicles was measured by the $Na^+$-dependent uptake of D-[U-$^{14}$C]glucose; at 15 s of incubation the overshoot compared to equilibrium was 25–40 fold. The vesicles were stored in liquid nitrogen up to 4 weeks without loss of transport or enzymatic activity.

*Uptake measurements.* The uptake of cephalexin, D-[U-$^{14}$C]glucose and L-[3-$^3$H]alanine was measured by the membrane filtration method [34,35] as described [8,12,15,32]. The concentration of cephalexin in the incubation mixtures was 2 mM. For uptake studies with L-[3-$^3$H]alanine 1 μCi of the radiolabeled compound, adjusted to a concentration of 100 μM with unlabeled L-alanine was used for each determination. Uptake studies with D-glucose were performed with 1 μCi D-[U-$^{14}$C]glucose corresponding to a final concentration of 19.34 μM of D-glucose for each determination. The composition of the incubation media is given in the legends to figures. The transport studies were carried out at 30 °C and initiated by mixing of 20 μl of brush-border membrane vesicles (100 μg of protein) with 180 μl of medium containing the substrate. After 1 min of incubation, transport was stopped by the addition of 1 ml of ice-cold stop solution (10 mM Tris-Hepes buffer (pH 7.4)/140 mM KCl) and the suspension was immediately pipetted on to the middle of a prewetted filter. After washing with 5 ml of ice-cold stop solution, the cephalexin taken up by the vesicles was determined by HPLC after extraction of the filters with water, using

a Waters HPLC chromatography system (Waters 840 Chromatography Data Station, Waters 490 E Programmable Multiwavelength Detector, WISP Model 712 Sample Processor, Model 510 HPLC pumps). Analysis was performed in a Bischoff 250 × 4 mm column (Bischoff, Leonberg, F.R.G.) filled with LiChrosorb RP 18 (7 μm, Merck, Darmstadt, F.R.G.) using a mixture of solvent A (30 mM sodium phosphate buffer (pH 6.2)/10 mM tetraethylammonium chloride) and solvent B (400 g of solvent A and 468 g of acetonitrile) at a ratio (v/v) of 73% solvent A and 27% solvent B. For the determination of radioactively labeled substrates the filters were dissolved in 4 ml of scintillator Quickzint 361. Radioactivity was measured by liquid scintillation counting in a Packard TriCarb 2000 counter (Packard, Downers Grove, IL, U.S.A.); corrections were made for quenching and eventual chemiluminescence. Each transport experiment was performed at least three times with different membrane preparations. The uptake values for cephalexin are determined in triplicate and are given as mean ± S.D.

*Chemical modification of brush-border membrane vesicles with amino acid reagents.* For treatment of brush-border membrane vesicles with the various reagents specific or selective for distinct amino acids, brush-border membrane vesicles preloaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were incubated with the different agents in the concentration range of $10^{-6}$ to $10^{-2}$ M at 20 °C for 1 h. The incubations with DCCD, DFP, DTNB, DTT, $HgCl_2$, NBD-chloride, NEM, PAO, PGO, phenazine methosulfate, PHMB, PITC, PMSF and TNBS were performed in 20 mM potassium phosphate buffer (pH 7.4)/300 mM mannitol. For modification of tyrosine residues with *N*-acetylimidazole the vesicles were diluted with 30 ml of 20 mM potassium phosphate buffer (pH 7.4)/140 mM NaCl. After centrifugation the vesicles were resuspended in the above-mentioned buffer and incubation with *N*-acetylimidazole was performed in the same buffer. The modification with DEP was performed at pH 6.4 in 20 mM potassium phosphate buffer/280 mM mannitol. For the modification with DFP a 50 mM stock solution of DFP in dimethylsulfoxide was prepared from DFP on Kieselgur; corresponding amounts of this stock solution were used. Some of the reagents had to be solved in dimethylsulfoxide or ethanol. The corresponding controls were incubated with equivalent volumes of the organic solvent, the concentration of the solvents was kept below 5%. After 1 h of incubation at 20 °C the vesicle suspension was diluted 50-fold with ice-cold 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol. After centrifugation the washing procedure was repeated. The final pellet was resuspended in 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol and used for the transport and photoaffinity labeling studies.

*Photoaffinity labeling.* Photoaffinity labeling of brush-border membrane vesicles was performed as described [8,12,15]. Typically, 200 μg of brush-border membrane protein (20 μl) preloaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol were mixed with 180 μl of 20 mM citrate-Tris buffer (pH 6.0)/140 mM KCl containing 10 μM (3 μCi) of 3-[*phenyl*-4-$^3$H]azidocephalexin, 0.15 μM (1.5 μCi) *N*-(4-azido[3,5-$^3$H]benzoyl)cephalexin or 1.25 μM (5 μCi) [$^3$H]benzylpenicillin. After 2 min of incubation in the dark at 20 °C the suspension was irradiated at 300 nm for 5 min with 3-[*phenyl*-4-$^3$H]azidocephalexin or at 254 nm for 30 s with *N*-(4-azido[3,5-$^3$H]benzoyl)cephalexin and for 120 s at 254 nm with [$^3$H]benzylpenicillin as photoprobe in a photochemical reactor Rayonet RPR 100 (Southern Ultraviolet, Hamden, CT, U.S.A.) equipped with 16 RPR 3000 Å or RPR 2543 Å lamps. Afterwards, 1 ml of ice-cold 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol/4 mM PMSF/4 mM iodacetamide/4 mM EDTA was added and the membranes were collected by centrifugation at 48000 × *g* for 30 min. After resuspension of the pellet in 200 μl of water, proteins were precipitated and submitted to SDS polyacrylamide gel electrophoresis as described [8,12,15,36].

## Results and Discussion

The active transport of polar and charged organic molecules across biological membranes is mediated by specific membrane carrier proteins. Specific interactions of amino acid residues in the respective transport protein with distinct functional groups of the substrate molecule are responsible for the specificity and efficacy of binding and transport. The knowledge about these interactions has important implications for the design of compounds or drugs being either permeable or impermeable to the plasma membrane of a distinct cell type. A promising approach for the investigation of such molecular interactions between a substrate molecule and its transport system is the chemical modification of the amino acid side-chains involved in the substrate–protein interaction. According to their functional groups in the side-chain the natural occurring amino acids can be classified into 4 groups: I, nonpolar hydrophobic amino acids; II, polar amino acids; III, acidic amino acids; and IV, basic amino acids. The functional moiety of polar and charged amino acids can be modified by chemical agents, the so-called group-specific reagents. Some of these reagents are not specific for only one amino acid but show a selectivity for certain amino acids. The study of the effects of a spectrum of group-specific reagents on a transport system should allow to establish a picture of the amino acid residues involved in the molecular interactions of the substrate and the transport protein.

In the present study we investigated the effect of chemical modification of reactive amino acid side-chains

on the transport activity of the $H^+$-dependent intestinal uptake system shared by small peptides and β-lactam antibiotics. In all the experiments performed we also measured the effect of the respective chemical modification on two other transport systems located in the brush-border membrane, the $Na^+$-dependent D-glucose transporter [37] and the $Na^+$-dependent L-alanine uptake system [38]. These parallel experiments allow discrimination between inhibition of the intestinal peptide transport system by unspecific effects and modification of amino acids essential for transport. The uptake of small peptides and orally active α-amino-β-lactam antibiotics is stimulated by an inwardly directed $H^+$-gradient, whereas the uptake of D-glucose and L-alanine are $Na^+$-driven transport processes. It is therefore unlikely that identical amino acid side-chains are involved in all three transport systems; the three transport systems should therefore behave differently to a modification of a certain amino acid.

*Effect of amino acid modifying reagents on the uptake systems for β-lactam antibiotics/oligopeptides, D-glucose and L-alanine*

In order to identify the involvement of a certain functional amino acid in the uptake of cephalexin into small intestinal brush-border membrane vesicles, the membrane vesicles were incubated for 1 h with the differetn group-specific reagents dissolved in the appropriate buffers in the concentration range of $10^{-6}$ to $10^{-2}$ M. After washing of the vesicles, the uptake of cephalexin into these pretreated membrane vesicles was measured both in the presence and absence of an inwardly directed $H^+$-gradient. Uptake measurement in the presence and the absence of a $H^+$-gradient should allow the detection of an amino acid modification at the substrate binding site or at the $H^+$-binding site. The uptake of D-[U-$^{14}$C]glucose and L-[3-$^{3}$H]alanine was measured in the presence of an inwardly directed $Na^+$-gradient. Usually, the uptake reaction was stopped after 1 min of substrate incubation and the intravesicular accumulation of the respective substrates into control membrane vesicles and vesicles pretreated with reagents was measured. The results of the experiments are given in Table I.

Since thiol groups have been shown to be essential for many $H^+$-dependent solute transport systems [18,20–30] and especially for the dipeptide/proton cotransport system in rabbit kidney [29,30], it seemed probable that thiol and/or disulfide bonds are also involved in the intestinal peptide transport system.

TABLE I

*Effect of amino acid modifying agents on the transport systems for β-lactam antibiotics/oligopeptides, D-glucose and L-alanine in rabbit small intestinal brush-border membrane vesicles*

Brush border membrane vesicles were treated with amino acid modifying reagents for 1 h at 20 C in the concentration range of $10^{-6}$ M to $10^{-2}$ M in the appropriate buffers as described in Materials and Methods. After washing with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol the uptake of 2 mM cephalexin, 19.34 μM D-[U-$^{14}$C]glucose or 100 μM L-[3-$^{3}$H]alanine was measured for 1 min. For measurement of cephalexin uptake, brush-border membrane vesicles (100 μg, 20 μg) were mixed with 180 μl of either 20 mM citrate-Tris buffer (pH 6.0)/140 mM KCl (measurement in the presence of a $H^+$-gradient) or 10 mM Tris-Hepes buffer (pH 7.4)/100 mM mannitol/100 mM NaCl (measurement in the absence of a $H^+$-gradient) containing 2 mM cephalexin. For measurement of D-glucose and L-alanine uptake, brush-border membrane vesicles were mixed with 180 μl of 10 mM Tris-Hepes buffer (pH 7.4)/100 mM mannitol/100 mM NaCl containing the radioactively labeled substrates.

| Reagent | Selective for | Inhibition of | | | | | |
|---|---|---|---|---|---|---|---|
| | | Cephalexin uptake | | $Na^+$-dependent | | | |
| | | $+H^+$-gradient ($IC_{50}$) | $-H^+$-gradient ($IC_{50}$) | D-glucose uptake ($IC_{50}$) | | L-alanine uptake ($IC_{50}$) | |
| PHMB | SH | ø | ø | +++ | ($6\cdot10^{-5}$ M) | +++ | ($6\cdot10^{-5}$ M) |
| $HgCl_2$ | SH | ø | ø | +++ | ($7\cdot10^{-5}$ M) | +++ | ($6\cdot10^{-5}$ M) |
| NEM | SH | ø | ø | ++ | ($9\cdot10^{-4}$ M) | ++ | ($2\cdot10^{-3}$ M) |
| DTNB | SH | ø | ø | ++ | ($5\cdot10^{-3}$ M) | ++ | ($6\cdot10^{-3}$ M) |
| PAO | vicinal SH | ø | ø | ++ | ($7\cdot10^{-4}$ M) | ++ | ($8\cdot10^{-4}$ M) |
| DTT | disulfide | ø | ø | + | ($>10^{-2}$ M) | (+) | |
| Phenazine methosulfate | SH | + ($>10^{-2}$ M) | + ($>10^{-2}$ M) | ++ | ($7\cdot10^{-4}$ M) | ++ | ($5\cdot10^{-4}$ M) |
| PMSF | OH | ø | ø | ø | | ø | |
| DFP | OH | ø | ø | ø | | ø | |
| PITC | $NH_2$ | ø | ø | + | ($10^{-2}$ M) | + | ($>10^{-2}$ M) |
| TNBS | $NH_2$ | ø | ø | n.d. | | n.d. | |
| NBD-chloride | $NH_2$, SH | ø | ø | +++ | ($6\cdot10^{-5}$ M) | +++ | ($8\cdot10^{-5}$ M) |
| PGO | arginine | ø | ø | ø | | ø | |
| DCCD | COOH | ø | ø | + | ($3\cdot10^{-3}$ M) | + | ($10^{-3}$ M) |
| *N*-Acetylimidazole | tyrosine | +($>10^{-2}$ M) | + ($2\cdot10^{-2}$ M) | ø | | ø | |
| DEP | histidine | +++ ($5\cdot10^{-5}$ M) | +++ ($10^{-4}$ M) | + | ($7\cdot10^{-3}$ M) | (+) | ($8\cdot10^{-3}$ M) |

However, none of the thiol modifying agents used, PHMB, $HgCl_2$, DTNB or NEM, had any inhibiting effect on the peptide transport system, whereas the $Na^+$-dependent transport systems for D-glucose and L-alanine were very strongly inhibited under identical conditions.

Reagents specific for vicinal thiol groups (PAO) and disulfide bonds (DTT) also did not affect the activity of the intestinal peptide transporter; only with the lipophilic thiol-oxidizing agent phenazine methosulfate could an inhibition of cephalexin uptake be achieved. The intestinal uptake system for small peptides/β-lactam antibiotics is therefore, in contrast to the renal transporter, not very susceptible to the modification by thiol reagents [29,30]. This difference in the behavior of the intestinal and the renal peptide transport system supports other findings [15,32] that the intestinal and the renal transport systems for small peptides and β-lactam antibiotics are composed of different proteins despite similar specificities and similar molecular weights.

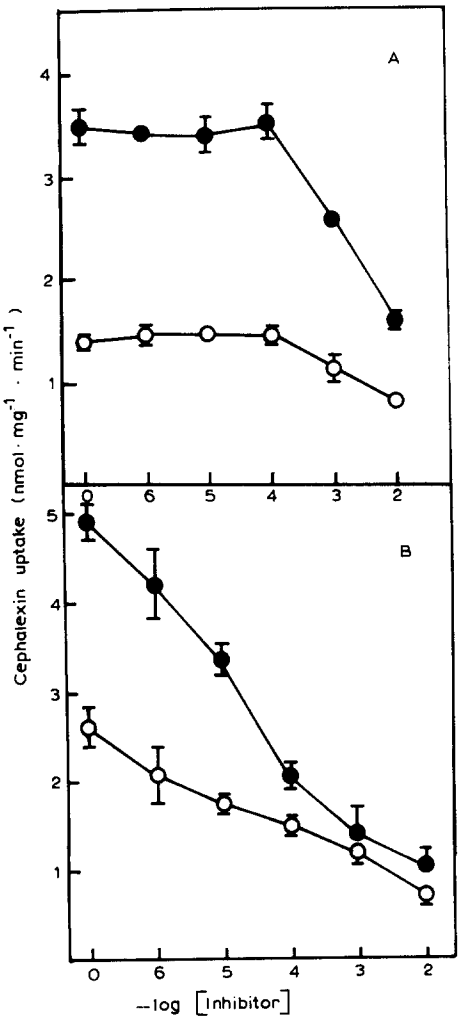


Fig. 1. Effect of *N*-acetylimidazole-treatment (A) and DEP-treatment (B) of brush-border membrane vesicles on the uptake of cephalexin. Brush-border membrane vesicles were treated with 0, $10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-3}$ and $10^{-2}$ M either of *N*-acetylimidazole in 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol (A) or DEP in 20 mM potassium phosphate buffer (pH 6.4)/280 mM mannitol (B) for 1 h at 20 °C. After washing the 1 min uptake of 2 mM cephalexin was measured in the presence (●) and the absence (○) of an $H^+$ gradient.

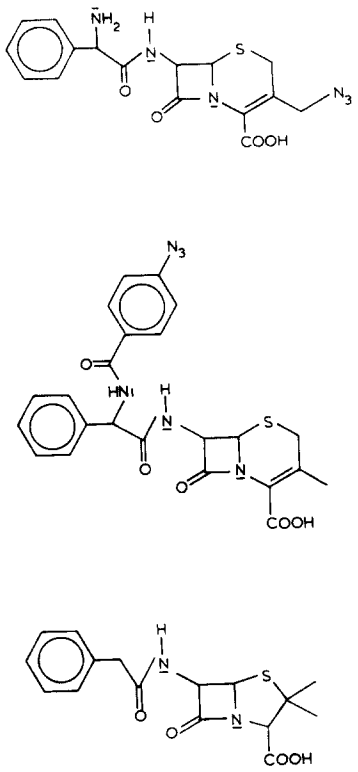


Fig. 2. Chemical structures of 3-azidocephalexin (top), *N*-(4-azidobenzoyl)cephalexin (middle) and benzylpenicillin (bottom).

The absence of essential thiol and disulfide groups in the transport system for β-lactam antibiotics and small peptides in small intestinal enterocytes explains the high stability of this transport system against photolytic damage during the photoaffinity labeling experiments with short-wavelength ultraviolet light. Irradiation of intestinal brush-border-membrane vesicles at 254 nm up to 3 min in the presence of oxygen did not significantly inhibited the $H^+$-dependent peptide transport system. In contrast, the $Na^+$-dependent D-glucose transport system is very sensitive to photolysis; irradiation even at 350 nm and after degassing of the vesicles with argon led to a progredient inactivation of the transporter activity [33].

Serine residues are often involved in enzymatic catalysis; a modification of amino acid hydroxyl groups with organic reactive fluorocompounds such as PMSF or DFP also showed no effect on the uptake of cephalexin. Furthermore, a chemical modification of amino groups with PITC, TNBS or NBD-chloride as well as of arginine residues with PGO [39] left the peptide transporter activity completely unchanged.

The transported substrates of the intestinal peptide transport system – di-, tripeptides and α-amino-β-lactam antibiotics – are amphoteric molecules carrying a positively charged $NH_3^+$-group and a negatively charged $COO^-$-group at physiological pH values. These groups may interact with functional groups of the protein capable of acting as general acids or general bases. Functional groups with characteristics for general acid/base catalysis are the carboxyl, the thiol, the phenolic hydroxyl, the amino and the imidazole groups.

Carboxyl groups of the acidic amino acids, aspartic acid and glutamic acid, in proteins can be modified with carbodiimides [40]. Treatment of brush-border membrane vesicles with DCCD had no influence on the peptide transporter.

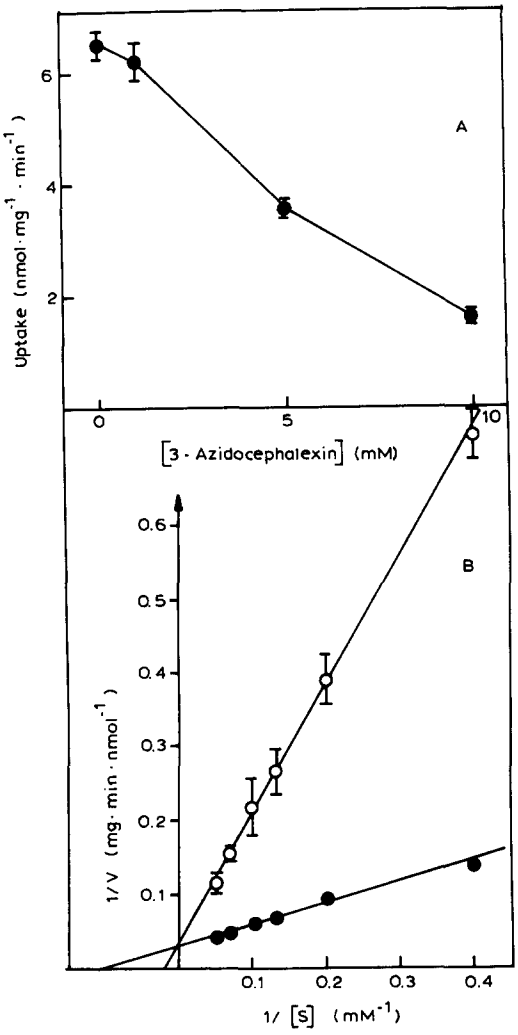


Fig. 3. Inhibition of cephalexin uptake into rabbit small intestinal brush-border membrane vesicles by 3-azidocephalexin. (A) The uptake of 2 mM cephalexin dissolved in 20 mM Tris-citrate buffer (pH 6.0)/140 mM HCl into brush-border membrane vesicles preloaded with 10 mM Tris-Hepes buffer (pH 7.4)/300 mM mannitol was measured for 1 min at 30 °C in the presence of 0, 1, 5 and 10 mM 3-azidocephalexin. (B) Kinetic characterization: The uptake of 2.5, 5, 7.5, 10, 15 and 20 mM cephalexin into brush-border membrane vesicles was measured for 1 min at 30 °C in the absence (●) and the presence (○) of 10 mM 3-azidocephalexin and analyzed by a Lineweaver-Burk diagram.

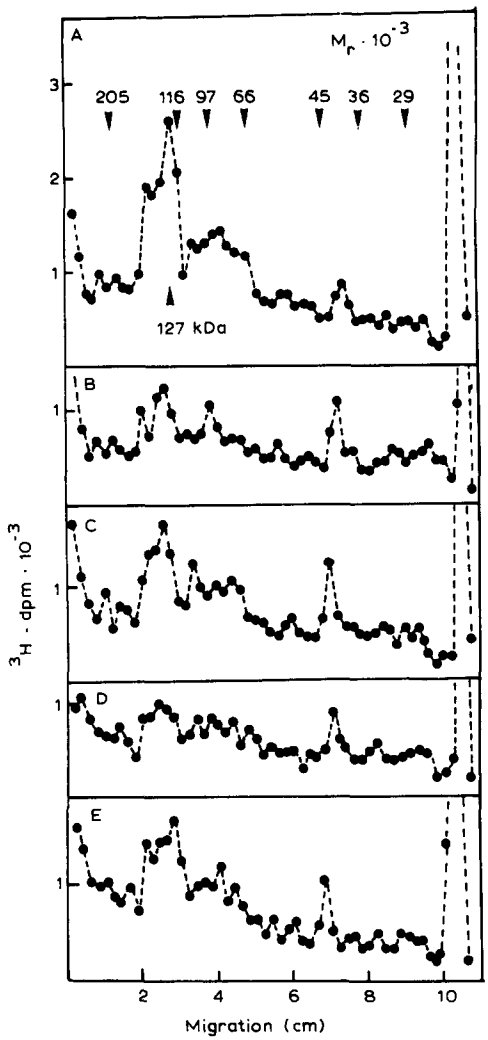


Fig. 4. Photoaffinity labeling of rabbit small intestinal brush-border membrane vesicles with 3-[*phenyl*-4-$^3$H]azidocephalexin and the effect of β-lactam antibiotics and dipeptides. Brush-border membrane vesicles (200 μg) were photolabeled with 10 μM (3 μCi) 3-[*phenyl*-4-$^3$H]azidocephalexin in the absence (A) and in the presence of 100 μM cephalexin (B), 100 μM benzylpenicillin (C) 2.5 mM L-carnosine (D) and 2.5 mM glycyl-L-proline (E). After washing the membrane proteins were separated by SDS-polyacrylamide gel electrophoresis on 7.5% gels.

Tyrosine residues in proteins can be selectively modified by acetylation using the reagent *N*-acetylimidazole [41]. Incubation of brush-border membrane vesicles with *N*-acetylimidazole led to a concentration-dependent inhibition of the uptake system for peptides and β-lactam antibiotics as is evident from Fig. 1A. The uptake of cephalexin was inhibited by concentrations of *N*-acetylimidazole greater than $10^{-4}$ M both in the presence and in the absence of an $H^+$-gradient. In contrast to the peptide transport system, the $Na^+$-dependent uptake systems for D-glucose and L-alanine remained unaffected. The inhibition of the intestinal peptide

transport system with *N*-acetylimidazole distinguishes the intestinal system from the dipeptide transport system in rabbit kidney, since the renal transport system was not affected in its transport activity by *N*-acetylimidazole [29]. These findings further support the hypothesis of different transport systems for small peptides in the small intestine and in the kidney [15,32].

Histidine residues in proteins can be selectively converted to *N*-carbethoxyimidazolyl residues at pH 6.4 by incubation with DEP. Fig. 1B shows that the uptake system for small peptides/β-lactam antibiotics is inhibited by DEP in a concentration-dependent manner, both in the presence and absence of an inwardly directed pH-gradient. The inhibitory effect of DEP on the transport system also in the absence of an $H^+$-gradient suggests a modifification of the binding site for β-lactam antibiotics/oligopeptides rather than a modification of the $H^+$-binding site. The inhibitory effect of DEP was nearly complete after 10 min of incubation as with the other reagents; for comparison with the other reagents the membrane vesicles were usually incubated with DEP for 1 h. As outlined previously in detail [32], the inhibition of the intestinal peptide transporter was apparently competitive with a 3–5-fold increase in the $K_m$ value in contrast to the non competitive inhibition of the renal transport system [29].

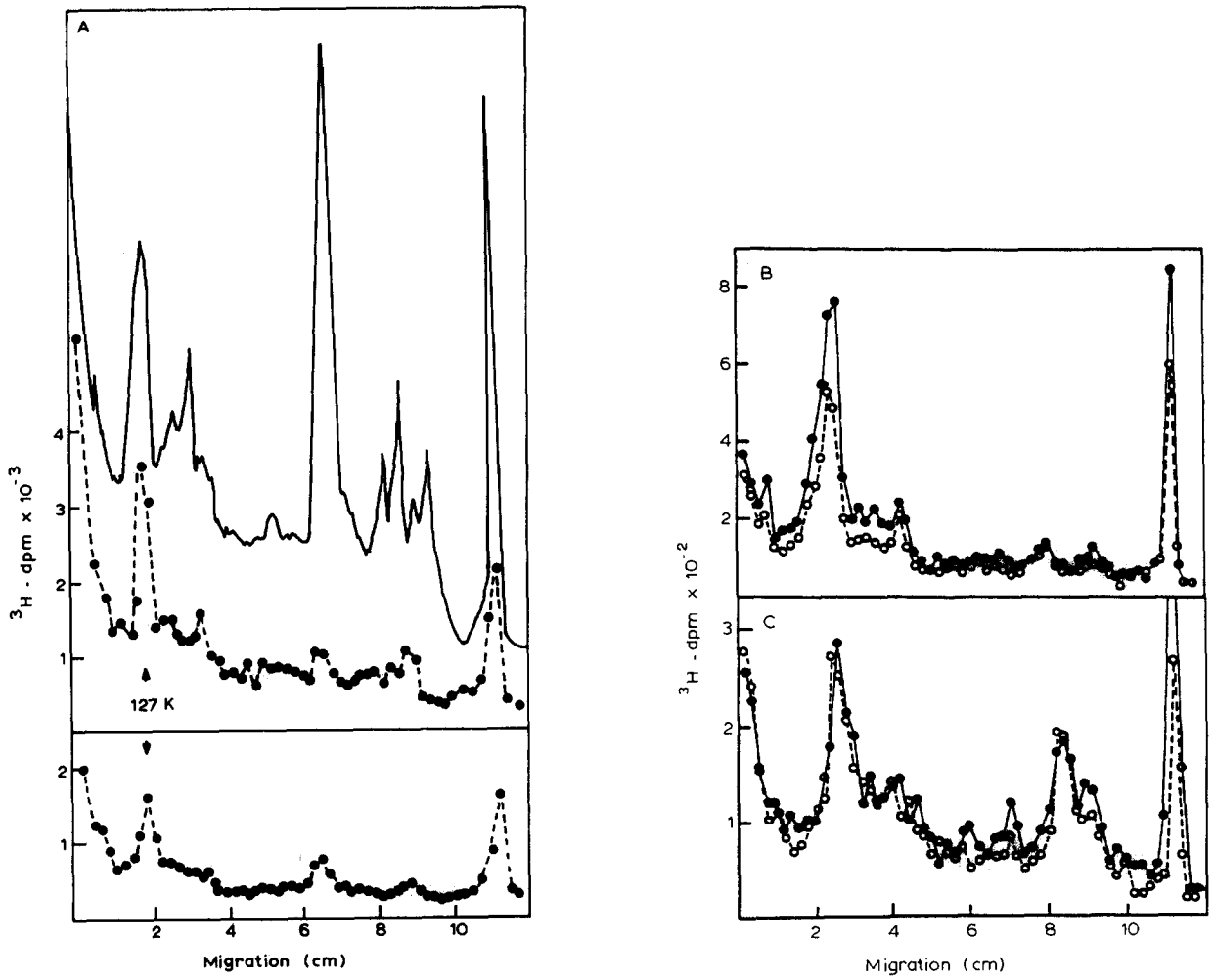


Fig. 5. Effect of DEP-treatment on photoaffinity labeling of the putative intestinal peptide transport protein. Brush-border membrane vesicles were treated with 10 mM DEP in 20 mM potassium phosphate buffer (pH 6.4)/280 mM mannitol/0.9% ethanol or for controls in the same buffer without DEP for 20 min at 20°C. After washing with 10 mM Tris-Hepes (pH 7.4)/300 mM mannitol, control and DEP-treated brush border membrane vesicles (250 μg of protein) were incubated with 10 μM (3 μCi) 3-[*phenyl*-4-$^3$H]azidocephalexin, 0.15 μM (1.5 μCi) *N*-(4-azido[3,5-$^3$H]benzoyl)cephalexin or 1.25 μM (5 μCi) [$^3$H]benzylpenicillin in 20 mM Tris-citrate buffer (pH 6.0)/140 mM KCl for 5 min in the dark. Photoaffinity labeling was performed for 5 min at 300 nm for 3-[*phenyl*-4-$^3$H]azidocephalexin, 30 s at 254 nm for *N*-(4-azido[3,5-$^3$H]benzoyl)cephalexin and 120 s at 254 nm for [$^3$H]benzylpenicillin. After washing of the vesicles, membrane polypeptides were separated by SDS-polyacrylamide gel electrophoresis on 7.5% gels. (A) Photolabeling with 3-[*phenyl*-4-$^3$H]azidocephalexin. The drawn line shows the distribution of Serva Blue R 250 stained polypeptides whereas the dotted line shows the distribution of radioactivity. In the upper graph control vesicles were separated, in the lower graph DEP-treated vesicles. (B) Photolabeling with [$^3$H]benzylpenicillin. ●, control; ○, DEP-treated vesicles. (C) Photolabeling with *N*-(4-azido[3,5-$^3$H]benzoyl)cephalexin. ●, control; ○, DEP-treated vesicles.

*Effect of DEP-treatment of rabbit small intestinal brush-border membrane vesicles on photoaffinity labeling of the putative peptide transport protein.* Kinetic experiments with various β-lactam antibiotics revealed that an interaction of the α-amino group in orally active α-amino-β-lactam antibiotics and a histidine residue at the transport site are obviously involved in the translocation of a carrier-bound substrate of the intestinal peptide transporter across the intestinal brush-border-membrane [31,32]. A decreased affinity of the intestinal peptide transport system for α-amino-β-lactam antibiotics obtained by DEP-treatment should be reflected by a decrease in the extent of photoaffinity labeling studies with 3-[*phenyl*-4-$^3$H]azidocephalexin (Fig. 2), a new photoreactive derivative of cephalexin still containing the α-amino group essential for oral activity of β-lactam antibiotics.

3-Azidocephalexin is in its antibacterial activity very similar to cephalexin after oral application [42] indicating an oral availability of 3-azidocephalexin. For a further kinetic characterization of this new photoprobe, we have performed transport studies using brush border membrane vesicles. The $H^+$-stimulated uptake of cephalexin is inhibited by 3-azidocephalexin in a concentration-dependent manner; kinetic analysis of this inhibition revealed as expected a competitive inhibition (Fig. 3). Photolysis of 3-[*phenyl*-4-$^3$H]azidocephalexin incubated with brush border membrane vesicles at 300 nm resulted in a covalent labeling predominantly of a 127 kDa binding protein (Figs. 4 and 5). Competition labeling experiments performed in the presence of unlabeled substrates indicated an identical specificity for binding as with [$^3$H]benzylpenicillin, *N*-(4-azido[3,5-$^3$H]benzoyl)cephalexin and *N*-(4-azido[3,5-$^3$H]benzoyl-(glycyl-L-proline; β-lactam antibiotics and dipeptides showed a protection from photoaffinity labeling (Fig. 4) whereas bile acids, amino acids or glucose had no effect. 3-Azidocephalexin is therefore a well suited photoreactive analogue of the orally active α-aminocephalosporin cephalexin. Since the affinity of the peptide transporter for cephalexin was greatly decreased by modification with DEP, the effect of DEP-treatment on photoaffinity labeling of the putative transport protein of $M_r$ 127 000 with different photoprobes was investigated. Photoaffinity labeling of intestinal brush-border membrane vesicles pretreated with DEP with 3-[*phenyl*-4-$^3$H]azidocephalexin resulted in a decrease in the extent of labeling of the 127 kDa binding protein for β-lactam antibiotics/oligopeptides compared to controls (Fig. 5A). With [$^3$H]benzylpenicillin as a direct photoaffinity probe for peptide transport systems [12] only a small decrease in the labeling of the 127 kDa polypeptide occurred by DEP-treatment (Fig. 5B). The labeling patterns between control and DEP-treated vesicles with *N*-(4-azido[3,5-$^3$H]benzoyl)cephalexin as photoprobe were nearly identical (Fig. 5C). These findings are consistent with the kinetic experiments; a modification of histidine residues in the transport protein inhibited the uptake and the efflux of β-lactam antibiotics with an α-amino group, whereas the uptake and the efflux of β-lactam antibiotics without an α-amino group was not significantly inhibited [32]. In the photoaffinity labeling experiments also, only the photoprobe with an α-amino group, 3-[*phenyl*-4-$^3$H]azidocephalexin, led to a significant decrease in the extent of labeling of the 127 kDa protein. These findings indicate a decreased affinity of the binding protein for the photoprobe and support the hypothesis that the interaction of the α-amino group in orally active α-amino-β-lactam antibiotics with a histidine residue of the transport system is essentially involved in the resorption process.

From the carried out, it is evident that out of the different amino acids occurring in proteins, only histidine and tyrosine residues are essential for the activity of the $H^+$-dependent transport system for peptides and β-lactam antibiotics in the enterocyte brush-border-membrane. Further studies with the purified transport protein and sequence are necessary to elucidate the three-dimensional molecular interactions of these amino acids with a carrier-bound substrate.

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