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DIRECT PHOTOAFFINITY LABELLING OF BINDING PROTEINS FOR β-LACTAM ANTIBIOTICS IN RABBIT INTESTINAL BRUSH BORDER MEMBRANES WITH [^{3}H]BENZYLPENICILLIN

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Abstract—Brush border membrane vesicles from rabbit small intestine were used to study the intestinal uptake system for β-lactam antibiotics. Benzylpenicillin inhibited the H^+-dependent uptake of α-aminocephalosporins in a concentration-dependent manner suggesting a common transport system for α-aminocephalosporins and benzylpenicillin. Benzylpenicillin is therefore a suitable probe to characterize this transport system. Irradiation of [^{3}H]benzylpenicillin using light sources having their maximum of radiation at 300 or 254 nm resulted in a covalent incorporation of radioactivity into penicillin binding proteins as was shown with serum albumin. Hence [^{3}H]benzylpenicillin can be used for direct photoaffinity labeling of penicillin binding proteins in different cells and tissues.

In brush border membrane vesicles from rabbit small intestine predominantly a membrane polypeptide with an apparent molecular weight of 127,000 was labeled by [^{3}H]benzylpenicillin. Competition labeling experiments demonstrated that β-lactam antibiotics—penicillins and cephalosporins—specifically interact with this protein, whereas amino acids, sugars or bile acids had no effect on the labeling pattern. Compounds which decreased the labeling of the 127,000 molecular weight membrane polypeptide also inhibited the H^+-dependent uptake of the α-aminocephalosporin cephalexin into intestinal brush border membrane vesicles. These results suggest that a polypeptide of molecular weight 127,000 in the brush border membrane from rabbit small intestine is a constituent of a common transport system responsible for the uptake of orally effective β-lactam antibiotics and dipeptides. β-Lactam antibiotics which are not absorbed from the small intestine also bind from the luminal site to this transport system, but are not transported across the brush border membrane.

β-Lactam antibiotics are absorbed from the gastrointestinal tract to a variable extent depending on their structure. Zwitterionic β-lactam antibiotics of low lipid solubility—aminopenicillins and aminocephalosporins—are very efficiently taken up by the small intestine [1–11]. Most β-lactam antibiotics of the third generation with high antibacterial activity are insufficiently absorbed from the gastrointestinal tract and must therefore be applied parenterally [12]. The polar α-aminocephalosporins are taken up by the intestinal dipeptide transport system [4, 7–9], whereas β-lactam antibiotics without an α-amino group are thought to be absorbed by simple diffusion processes across the intestinal brush border membrane [12–15].

To investigate the molecular mechanisms responsible for the intestinal absorption of β-lactam antibiotics, brush border membrane vesicles from rabbit small intestine were used for photoaffinity labeling and transport studies. In the present study we could demonstrate that benzylpenicillin interacts with the intestinal uptake system for α-aminocephalosporins and dipeptides. Direct photoaffinity labeling of rabbit small intestinal brush border membrane vesicles with [^{3}H]benzylpenicillin identified a membrane polypeptide of molecular weight 127,000, which may be a component of the intestinal uptake system for β-lactam antibiotics and dipeptides in the brush border membrane. Part of the results have been reported in preliminary form [16, 17].

MATERIALS AND METHODS

Materials. [Phenyl-4-(*n*)-^{3}H]benzylpenicillin (specific radioactivity 8–31 Ci/mmol) was obtained from Amersham (Amersham Buchler GmbH & Co KG, Braunschweig, F.R.G.). β-Lactam antibiotics were obtained from Sigma (Sigma, München, F.R.G.) and other biochemicals and materials for electrophoresis were from Serva (Serva, Heidelberg, F.R.G.). Solvents for high pressure liquid chromatography were from Merck (Merck, Darmstadt, F.R.G.) and all other substances were from commercial sources and of highest purity available.

Preparation of brush border membrane vesicles from the small intestine. White rabbits (3–3.5 kg, Tierzucht Kastengrund, Hoechst Aktiengesellschaft Frankfurt am Main, F.R.G.) were killed by an intravenous injection of 0.5 ml of an aqueous solution containing 2.5 mg tetracain-HCl, 100 mg embutramid and 25 mg mebezonium iodide. The small intestines were immediately removed and rinsed with ice-cold phosphate buffered saline. The intestines were cut into pieces of 30 cm length which were

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frozen at −80° until preparation of membrane vesicles. For the preparation of brush border membrane vesicles intestinal segments were thawed at 37° and the mucosa was scraped off. The mucosal scrapings were homogenized for 2 min with an Ultra-Turrax homogenizer (IKA Werk, Staufen, F.R.G.) and the brush border membrane vesicles were isolated by the Mg^{2+}-precipitation method as described [18]. The final pellet of the vesicle preparation was suspended in the desired volume of 300 mM mannitol, 10 mM Tris/Hepes, pH 7.4 with the aid of a syringe and a 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 ± 5× and 23 ± 4×, respectively. Protein was determined according to Bradford [19] using the Bio-Rad kit (Bio-Rad, München, F.R.G.). Leucine aminopeptidase and γ-glutamyltransferase were tested using the Merckotest kits 3359 and 3394 (Merck, Darmstadt, F.R.G.). The quality of the vesicles was tested by measurement of Na^+-dependent D-glucose uptake. The overshoot at 15 sec compared to equilibrium was 24–35×. The vesicles were stored in liquid nitrogen up to 4 weeks without any loss of Na^+-dependent D-glucose transport or enzyme activities. Brush border membrane vesicles from rat or pig small intestines were prepared in the same way from freshly excised small intestines.

Uptake measurements. Uptake of [^{3}H]benzylpenicillin, [^{14}C]D-glucose and unlabeled cephalexin was measured by the membrane filtration technique [20, 21]. The composition of the media is given in the legends to figures. Transport studies were carried out at 30°. Individual tubes were used for each time point. Usually, 20 μl of vesicle suspension (100–150 μg) were pipetted as a drop on the wall of disposable polystyrene tubes (11 × 70 mm) containing 180 μl of medium with the respective ligands. Transport was initiated by stirring on a Vortex mixer for 2 sec. At the desired time transport was stopped by addition of 1 ml of ice-cold stop solution (150 mM KCl, 10 mM Tris/Hepes, pH 7.4). This mixture was immediately pipetted onto the middle of a pre-wetted filter (cellulose nitrate, Millipore HAWP 0.45 μm, Millipore, Eschborn, F.R.G.) kept under suction (25–35 mbar). The filter was washed with 5 ml of ice-cold stop solution. For uptake measurements with radioactively labeled substrates the filters were dissolved with 4 ml of scintillator Quickszint 361 (Zinsser Analytic GmbH, Frankfurt, F.R.G.) and radioactivity was determined by liquid scintillation counting. For uptake measurements with unlabeled cephalexin a disc of 1 cm diameter containing the vesicles was cut from each filter and the cephalosporin was extracted with 300 μl of water [22]. 200 μl of each probe were analyzed by high pressure liquid chromatography using a Waters chromatography system (M 740 data module, M 490 detector, M 680 automated gradient controller, M 501/510 HPLC pumps, M 712 automated sample processor, Waters Instruments, Eschborn, F.R.G.). A Bischoff 250 × 4 mm-column (Bischoff, Leonberg. F.R.G.) filled with LiChrosorb RP 18, 7 μm (Merck, Darmstadt, F.R.G.) was used and cephalexin was eluted with 16.4% acetonitrile/83.6% 30 mM sodium phosphate buffer, pH 7.0 at a flow rate of 1 ml/min and detected at 262 nm. The retention time for cephalexin under these conditions was 4.3 min. No corrections for filter binding of cephalexin had to be made, since no more than 5 pmol were unspecifically bound to the cellulose nitrate filters.

Photoaffinity labeling. Prior to photoaffinity labeling the desired amount of [^{3}H]benzylpenicillin (in acetonitrile/20 mM sodium phosphate buffer, pH 7.8, (3/7, v/v)) was removed from the stock solution, dried in the vacuum and redissolved in 100 mM sodium phosphate buffer pH 7.4. For photoaffinity labeling of albumin 10 μCi of [^{3}H]benzylpenicillin in 300 μl buffer were mixed with 200 μl of a solution of human serum albumin in 100 mM sodium phosphate buffer, pH 7.4 (final concentration: 0.4 mg/ml) in a cell culture dish (30 mm diameter). Photolysis was carried out in a Rayonet photochemical reactor RPR 100 (The Southern Ultraviolet Company, Hamden, CT, U.S.A.) equipped either with 16 RPR 2530 Å, 16 RPR 3000 Å or 16 RPR 3500 Å lamps at a distance of 15 cm from the lamps (according to data of the manufacturer the RPR 2530 Å lamps emit 84% of their radiation at 2530 Å with an intensity of 12,800 μW/cm^2 and 1.65×10^{16} photons sec^{-1} cm^{-3}. The RPR 3000 Å lamps have an emission maximum at 3000 Å with a half-bandwidth of 300 Å and a photon intensity of 4×10^{17} sec^{-1} cm^{-3}. The RPR 3500 Å lamps have an emission maximum at 3500 Å with a half-bandwidth of 35 nm and a photon intensity of $1.5–5 \times 10^{16}$ photons sec^{-1} cm^{-3} and an irradiation intensity of 9200 μW/cm^2). For photoaffinity labeling with monochromatic light the probes were irradiated for 10 min with narrow-bandwidth light in a rectangular cuvette (1 × 10 mm) of quartz glass in the sample chamber of a Hitachi F-3000 fluorimeter (Hitachi Ltd, Tokyo, Japan) equipped with a 150 W xenon arc lamp. Wavelengths were selected with the excitation monochromator. After 0, 3, 9 and 15 min of irradiation 100 μl aliquots (40 μg) were removed and protein was precipitated with 500 μl of dioxane.

For photoaffinity labeling of brush border membrane vesicles 200–300 μg of vesicles were suspended in a total volume of 200 μl with 10 mM sodium phosphate buffer, pH 7.4 containing 140 mM NaCl and 2–4 μCi of [^{3}H]benzylpenicillin. After 5 min incubation in the dark at 20° the membrane suspension was transferred to cell culture dishes and photolyzed for 2 min at 254 nm as described above. After photolysis the suspension was diluted with 1 ml of 10 mM sodium phosphate buffer, pH 7.4 containing 4 mM phenylmethyl sulfonylfluoride, 4 mM iodacetamide and 4 mM EDTA [23] and centrifuged at 48,000 *g* for 30 min. The pellet was resuspended in 200 μl of water and protein was precipitated as described [24].

SDS gel electrophoresis. The dried protein precipitates were dissolved in 40 μl of 62.5 mM Tris/HCl buffer, pH 6.8 containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenolblue. After centrifugation at 15,000 *g* for 5 min the clear supernatants were submitted to discontinuous sodium dodecylsulfate gel electrophoresis on 0.7 × 200 × 150 mm slab gels using a Pharmacia LE 4/2 apparatus (Pharmacia, Freiburg, F.R.G.). After

electrophoresis the gels were fixed and stained as described [25]. After densitometry with a densitometer CD-50 (DESAGA, Heidelberg, F.R.G.) the gels were cut into 2 mm slices. Protein was digested with 0.25 ml of Biolute (Zinsser Analytic GmbH, Frankfurt, F.R.G.) and after addition of 4 ml scintillator Quickszint 501 (Zinsser Analytic GmbH, Frankfurt, F.R.G.) radioactivity was determined by liquid scintillation counting.

RESULTS

Interaction of benzylpenicillin with the intestinal transport system for α-aminocephalosporins

In order to investigate whether benzylpenicillin interacts with the uptake system for α-aminocephalosporins and dipeptides, the effect of benzylpenicillin on the carrier-mediated uptake of cephalexin into brush border membrane vesicles from rabbit small intestine was measured. Figure 1 shows that benzylpenicillin decreased the active H^+-dependent uptake ($pH_{out} = 6.0$, $pH_{in} = 7.4$) of cephalexin in a concentration-dependent manner (Fig. 1A). The kinetic analysis of this inhibition suggests a competitive inhibition of cephalexin uptake by benzylpenicillin (Fig. 1B). The uptake of benzylpenicillin into intestinal brush border membrane vesicles was inversely proportional to the medium osmolarity indicating that benzylpenicillin is transported across the brush border membrane into the intravesicular space (data not shown). Heating of brush border membranes to 70° prior to transport studies greatly decreased the uptake of cephalexin as well as of benzylpenicillin indicating that the transport of both compounds is mediated by a membrane protein (data not shown). The competitive inhibition of the carrier-mediated uptake of cephalexin by benzylpenicillin suggests that benzylpenicillin competes with the intestinal transport system for α-aminocephalosporins and dipeptides. Therefore, radioactively labeled photolabile derivatives of benzylpenicillin should be suitable probes for the characterization of the intestinal uptake system shared by α-aminocephalosporins and dipeptides [4, 7–9].

Suitability of [^{3}H]benzylpenicillin for photoaffinity labeling

To investigate whether [^{3}H]benzylpenicillin can be used for direct photoaffinity labeling, solutions of human serum albumin were incubated with [^{3}H]benzylpenicillin and irradiated with light sources having their maximum of irradiation at 254 nm, 300 nm or 350 nm. After desired times aliquots were removed and the covalent incorporation of benzylpenicillin into albumin was determined by sodium dodecylsulfate gel electrophoresis. Figure 2 shows that irradiation at 254 nm or 300 nm led to a high covalent attachment of the penicillin ligand to the albumin molecule, whereas no significant incorporation of radioactivity was obtained by irradiation at 350 nm. Photoaffinity labeling of albumin with [^{3}H]benzylpenicillin was also performed with monochromatic light at 220, 230, 240, 250, 260, 270, 280 and 300 nm. Figure 2B shows that a maximal labeling of albumin was obtained at 240 nm. These experiments prove that radioactively labeled benzylpenicillin can be directly used for photoaffinity labeling of penicillin binding proteins without modification of the ligand molecule by the introduction of photosensitive groups.

Identification of binding proteins for benzylpenicillin in brush border membrane vesicles

In order to identify binding proteins presumably involved in the intestinal uptake of β-lactam antibiotics, brush border membrane vesicles from rabbit

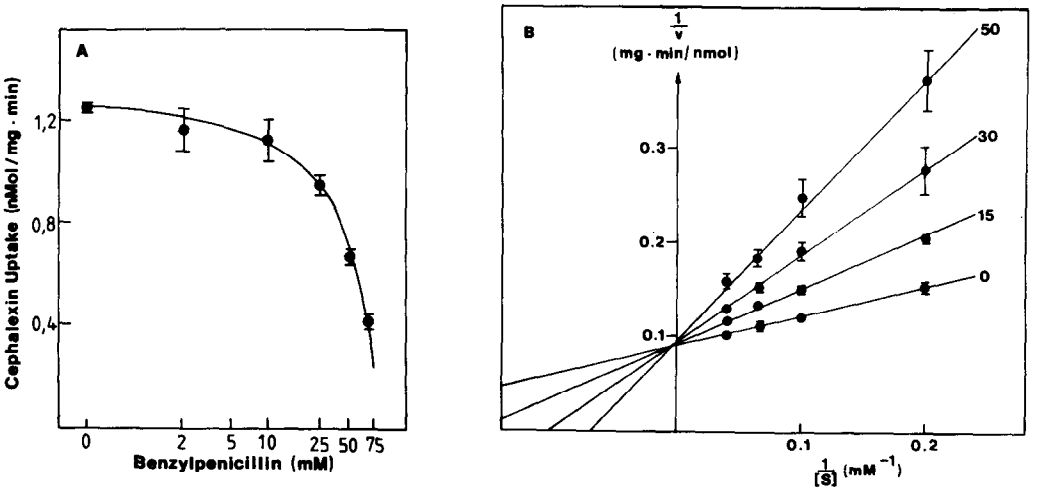

Fig. 1. Inhibition of cephalexin uptake into brush border membrane vesicles by benzylpenicillin. Rabbit small intestinal brush border membrane vesicles (100 μg, 20 μl) loaded with 10 mM Tris/Hepes buffer, pH 7.4, 300 mM mannitol were incubated at 30° for 1 min with 180 μl 10 mM citrate/Tris buffer, pH 6.0 140 mM KCl. (A) In the presence of 1 mM cephalexin and 0, 2, 10, 25, 50 and 75 mM benzylpenicillin. (B) The uptake of 5, 10, 15 and 20 mM cephalexin was measured in the absence and in the presence 15, 30 and 50 mM benzylpenicillin.

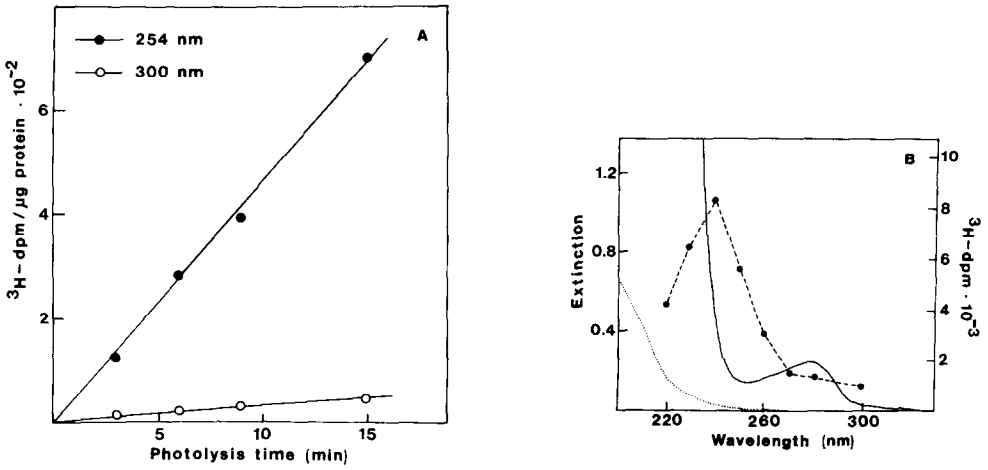

Fig. 2. Suitability of [^{3}H]benzylpenicillin for direct photoaffinity labeling. Human serum albumin (0.4 mg/ml) in 100 mM sodium phosphate buffer, pH 7.4 was incubated at 20° for 10 min in the dark with 0.32 μM (0.4 μCi/μg protein) (A) or 1.2 μM (3.75 μCi/μg protein) (B)[^{3}H]benzylpenicillin. (A) Photolysis was carried out in a Rayonet photochemical reactor RPR 100 with lamps having their maximum of radiation at 254, 300 or 350 nm for 0, 3, 6, 9 and 15 min. 40 μg Aliquots were removed after the desired times and submitted to SDS gel electrophoresis. (B) Photolysis was carried out with narrow bandwidth light in a Hitachi F 3000 fluorimeter for 10 min at 220, 230, 240, 250, 260, 270, 280 and 300 nm: . . . . . , absorption spectrum of benzylpenicillin (20 μM in water); ——, absorption spectrum of albumin (0.5 mg in 100 mM sodium phosphate buffer, pH 7.4; ●–●–●, incorporation of radioactivity into albumin after irradiation at the indicated wavelengths.

small intestine were incubated with [^{3}H]benzylpenicillin and submitted to photoaffinity labeling followed by sodium dodecylsulfate electrophoresis. Figure 3 shows a clear incorporation of radioactivity predominantly into one membrane polypeptide of apparent molecular weight 127,000. Incubation of the membrane vesicles with [^{3}H]benzylpenicillin in the dark without illumination did not result in any covalent labeling of membrane proteins indicating a photocatalyzed incorporation of benzylpenicillin into the labeled membrane polypeptides. Photoaffinity labeling of brush border membrane vesicles from rat or pig small intestine also resulted in a prominent labeling of a 127,000 molecular weight membrane polypeptide (data not shown). β-Lactam antibiotics may acylate proteins [26]. The release of the β-lactam antibiotics from the acylated penicillin binding proteins of bacteria is greatly accelerated by hydroxylamine and after 1 hr of incubation most of the covalently bound β-lactam antibiotics were released from the penicillin binding proteins [26]. In order to characterize the photocatalyzed product between benzylpenicillin and the respective binding proteins, brush border membrane vesicles photolabeled with [^{3}H]benzylpenicillin were incubated with hydroxylamine. Figure 4 shows that incubation of brush border membranes photolabeled with [^{3}H]benzylpenicillin with hydroxylamine did not change the labeling pattern. These data exclude the possibility that the covalent labeling of the penicillin binding polypeptide of molecular weight 127,000 is the result of an acylation reaction.

The specificity of the labeling by [^{3}H]benzylpenicillin was determined by photoaffinity labeling experiments in the presence of various compounds.

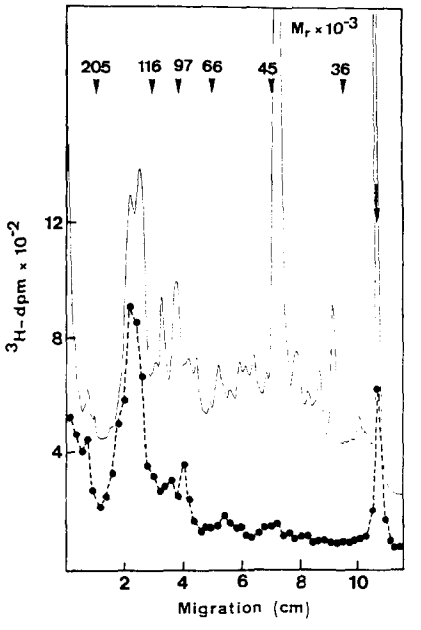

Fig. 3. Incorporation of radioactivity after SDS gel electrophoresis of rabbit small intestinal brush border membrane vesicles after photoaffinity labeling. 300 μg of brush border membrane vesicles from rabbit small intestine were photolabeled with 0.59 μM (2.64 μCi) [^{3}H]benzylpenicillin and subsequently submitted to SDS gel electrophoresis. Total acrylamide concentration was 7.5%. The upper curve shows the densitometer tracing of the Serva Blue R 250 stained polypeptides, whereas the lower curve indicates the distribution of radioactivity. The positions of the molecular weight marker proteins (M_r) and the tracking dye bromophenol blue are indicated by arrows.

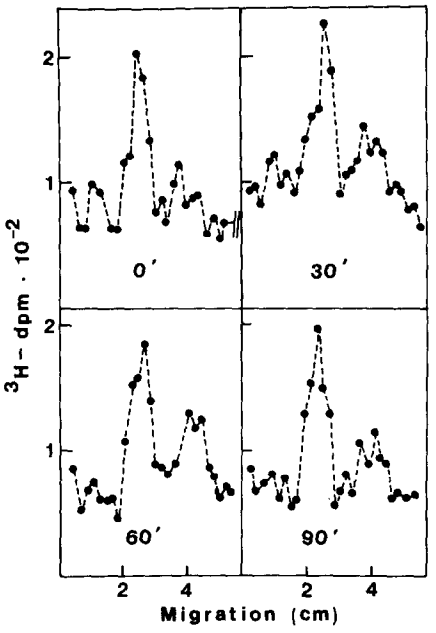

Fig. 4. Effect of hydroxylamine on the penicillin-binding polypeptides of rabbit small intestine brush border membranes photolabeled with [^{3}H]benzylpenicillin. Brush border membrane vesicles (1.5 mg) from rabbit small intestine were photolabeled with 0.53 μM (25 μCi) [^{3}H]benzylpenicillin. After washing of the vesicles, 250 μg of labeled membranes were incubated at 20° with 200 mM hydroxylamine in 100 mM sodium phosphate buffer, pH 7.4 for 0, 30, 60 and 90 min. Subsequently the membranes were washed and submitted to SDS gel electrophoresis on 7.5% gels.

Compounds which compete with [^{3}H]benzylpenicillin for the binding to specific membrane proteins should decrease the extent of labeling of the benzylpenicillin binding membrane polypeptides. Penicillins such as amoxicillin decreased the labeling of the benzylpenicillin binding proteins in a concentration-dependent manner (Fig. 5B). Orally effective α-aminocephalosporins such as cephalexin also showed a clear concentration-dependent inhibition of the labeling of the respective membrane polypeptide (Fig. 5C). Since cephalexin shares the intestinal transport system for dipeptides [4, 7–9] various dipeptides such as carnosine or glycyl-L-proline were also used for competition labeling experiments. Figure 6 demonstrates that dipeptides decreased the extent of labeling of the benzylpenicillin binding polypeptides in the small intestinal brush border membrane. Control experiments excluded that the decrease of labeling resulted from optical shielding. In these control experiments photoaffinity labeling was performed in rectangular cuvettes (1 mm thickness) and the irradiated light was filtered through a solution of the respective protective ligand (thickness 1 mm). Figure 7 shows that no significant decrease of labeling occurred if the irradiated light was filtered through a 100 μM solution of cephalexin of 1 mm thickness. However, if cephalexin was present in the vesicle suspension, a clear decrease in the extent of labeling of the 127,000 molecular weight polypeptide occurred (Fig. 7C). Furthermore, the ratio of radioactivity in the labeled polypeptide to radioactivity of background decreases in the presence of cephalexin. If optical shielding were responsible for the decrease in the extent of labeling, this ratio should remain constant. Similar results were obtained with amoxicillin and other penicillins (Fig. 7D and E) indicating that the decrease in the extent of labeling results from a competition of the respective protective ligands with [^{3}H]benzylpenicillin for the binding to the membrane protein. Substrates like the imino acid proline, the sugar D-glucose or the bile acid taurocholate, compounds which are taken up by other systems than the dipeptide transporter [25, 27–29], had no effect on the labeling of brush border membrane polypeptides by [^{3}H]benzylpenicillin (data not shown). The effect of various β-lactam antibiotics, penicillins as well as cephalosporins, on the labeling pattern is shown in Fig. 8. All β-lactam antibiotics decreased the labeling of the 127,000 molecular weight polypeptide, no matter whether the respective β-lactam antibiotic is enterally absorbed or not. This specificity of the benzylpenicillin binding membrane polypeptide of molecular weight 127,000 for the binding of compounds with dipeptide or tripeptide structure is in agreement with the specificity of the intestinal

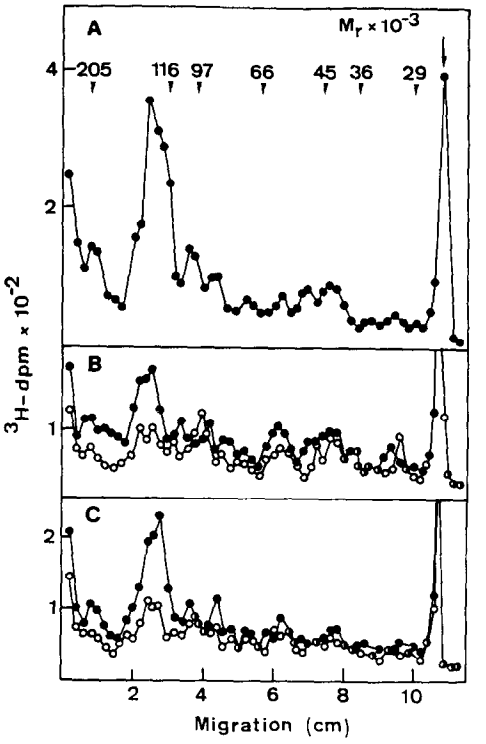

Fig. 5. Effect of amoxicillin and cephalexin on the labeling of benzylpenicillin binding polypeptides in the brush border membrane from rabbit small intestine. 200 μg of brush border membrane vesicles from rabbit small intestine were photolabeled with 0.41 μM (3.57 μCi) [^{3}H]benzylpenicillin: (A) in the absence of inhibitors; (B) in the presence of 100 (●) and 500 (○) μM amoxicillin; (C) in the presence of 10 (●) and 50 (○) μM cephalexin. Total acrylamide concentration was 7.5%. For all other conditions see legend to Fig. 5.

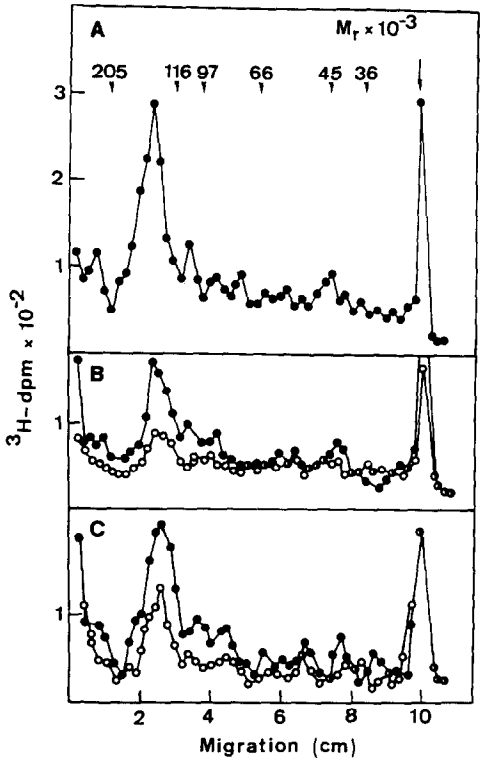

Fig. 6. Effect of dipeptides on the labeling of benzylpenicillin binding polypeptides in the brush border membrane from rabbit small intestine. 200 μg of brush border membrane vesicles from rabbit small intestine were photolabeled with 0.41 μM (3.57 μCi) [^{3}H]benzylpenicillin: (A) in the absence of inhibitors; (B) in the presence of 2.5 (●) and 10 mM (○) L-carnosine; (C) in the presence of 0.5 (●) and 2.5 (○) glycyl-L-proline. Total acrylamide concentration was 7.5%. For all other conditions see legend to Fig. 5.

uptake system for orally effective cephalosporins. The H^+-dependent uptake of the α-aminocephalosporin cephalexin was measured in the presence of various compounds. Table 1 shows that dipeptides clearly inhibited the uptake of cephalexin, whereas amino acids, glucose or bile acids had no influence. β-Lactam antibiotics—both penicillins and cephalosporins—inhibited the H^+-dependent cephalexin uptake. β-Lactam antibiotics which are not enterally absorbed such as cephaloridine, cefotiam, cefoperazone and others also inhibited the uptake of orally effective α-aminocephalosporins.

DISCUSSION

The extent of intestinal absorption of β-lactam antibiotics depends on the structure of the antibiotic molecule. The uptake of zwitterionic α-aminocephalosporins occurs by the intestinal transport system for dipeptides [4, 7–9]. β-Lactam antibiotics without an α-amino group such as benzylpenicillin are believed to be absorbed by simple diffusion processes through the lipid barrier of the intestinal brush border membrane [12–15]. Recent studies, however, have demonstrated that benzylpenicillin is transported into isolated hepatocytes [30, 31] and into the rat choroid plexus [32] by carrier-mediated uptake processes. In the present study we investigated whether benzylpenicillin interacts with the intestinal uptake system for α-aminocephalosporins and dipeptides. Benzylpenicillin inhibited the uptake of orally active α-aminocephalosporins in a concentration-dependent manner. This interaction of benzylpenicillin with the uptake of cephalexin suggests that benzylpenicillin is taken up into intestinal brush border membrane vesicles by the transport system for α-amino-β-lactam antibiotics and dipeptides rather than by simple diffusion processes. Benzylpenicillin is therefore a suitable probe for the characterization of this transport system. Since the binding of a transported ligand to protein components of the respective transport system preceeds the uptake process, the identification of putative binding proteins is an important step for the investigation of the transport mechanism.

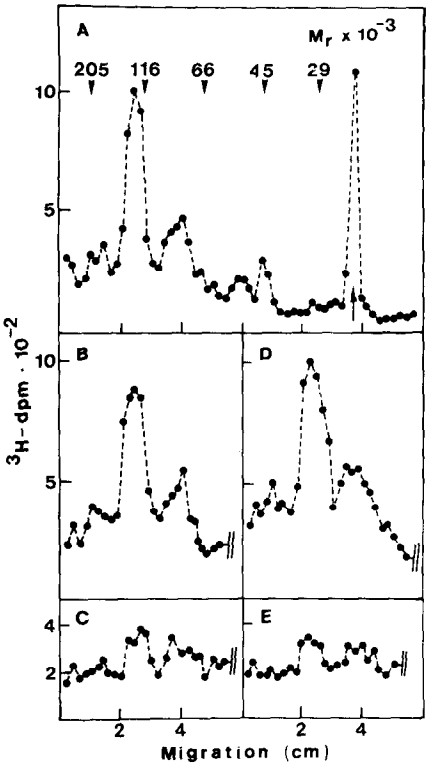

Fig. 7. Effect of optical shielding on the labeling of brush border membrane vesicles from rabbit small intestine by [^{3}H]benzylpenicillin in the presence of cephalosporins and penicillins. Brush border membrane vesicles (250 μg) from rabbit small intestine were photolabeled at 254 nm with 0.64 μM (5 μCi) [^{3}H]benzylpenicillin in quartz cuvettes (film thickness 1 mm) and subsequently submitted to SDS gel electrophoresis: (A) without filter solution and without protective ligands in the vesicle suspension; (B) with a 100 μM cephalexin filter solution and without cephalexin in the vesicle suspension; (C) with a 100 μM cephalexin filter solution and in presence of 100 μM cephalexin in the vesicle suspension; (D) with a 200 μM amoxicillin filter solution and without amoxicillin in the vesicle suspension; (E) with a 200 μM amoxicillin filter solution and in presence of 200 μM amoxicillin in the vesicle suspension.

Table 1. Effect of various compounds on the carrier-mediated uptake of cephalexin into brush border membrane vesicles from rabbit small intestine

Inhibitor	Cephalexin	%-Uptake
None		100
12.5 mM L-Proline	1 mM	93.9 ± 6.2
25 mM L-Proline	2 mM	95.6 ± 6.5
12.5 mM Glycine	1 mM	103 ± 9.6
25 mM L-Alanine	2 mM	100.9 ± 7.1
12.5 mM Glycyl-L-proline	1 mM	47.2 ± 1.3
25 mM Glycyl-L-proline	2 mM	51.4 ± 8.5
25 mM L-Prolyl-glycine	2 mM	69.8 ± 6.7
12.5 mM L-Carnosine	1 mM	46.5 ± 0.3
25 mM L-Carnosine	2 mM	55.3 ± 4.9
1 mM Taurocholate	2 mM	99.4 ± 1.8
12.5 mM Benzylpenicillin	1 mM	56.1 ± 8.1
25 mM Carbenicillin	2 mM	76.8 ± 5.4
10 mM Dicloxacillin	2 mM	53.9 ± 9.2
12.5 mM Cefadroxil	1 mM	38.4 ± 9.1
25 mM Cephradine	2 mM	60.8 ± 0.9
25 mM Ceftazidime	2 mM	78.1 ± 3.2
25 mM Cefoperazone	2 mM	60.6 ± 9.7
25 mM Cefotiam	2 mM	43.4 ± 0.5
25 mM Cephaloridine	2 mM	70.5 ± 7.3
12.5 mM Cephalothin	1 mM	54.9 ± 3.6

Brush border membrane vesicles (100 μg, 20 μl) from rabbit small intestine preloaded with 10 mM Tris/Hepes buffer, pH 7.4, 300 mM mannitol were incubated at 30° for 1 min with 180 μl of 10 mM citrate/Tris buffer, pH 6.0, 140 mM KCl containing cephalexin and the indicated substances. The control value for cephalexin uptake was 0.935 nmol/mg protein × min at 1 mM cephalexin and 2.042 nmol/mg protein × min at 2 mM cephalexin. Uptake in presence of inhibitors is expressed as percentage of the control ± SE.

Photoaffinity labeling has been successfully used for the identification of binding proteins and transport systems [33–35].

A prerequisite for the identification of transport proteins by photoaffinity labeling is the availability of suited photolabile derivatives of the respective ligands with high specific radioactivity. Radioactively labeled benzylpenicillin is commercially available and it seems possible that [^{3}H]benzylpenicillin can be covalently attached to proteins by irradiation with ultraviolet light. Photolysis of [^{3}H]benzylpenicillin with UV light at 254 nm in the presence of serum albumin revealed a considerable photocatalyzed covalent incorporation of radioactivity into the albumin molecule (Fig. 2). This covalent linkage between albumin and benzylpenicillin may be caused by two mechanisms: (a) generation of reactive intermediates of benzylpenicillin; (b) generation of reactive intermediates in the protein molecule [36–38]. By photoaffinity labeling with monochromatic light a maximal incorporation of radioactivity was achieved at 240 nm (Fig. 2B) where benzylpenicillin only poorly absorbs. Hence, photoaffinity labeling of penicillin binding proteins appears to proceed predominantly via activation of the protein. The maximal extent of photoaffinity labeling at 240 nm suggests that a phenylalanine residue of the binding protein may be activated. At lower wavelengths a direct activation of the benzylpenicillin molecule is possible, whereas an activation of tryptophan residues as in the case of the human erythrocyte D-glucose transporter [36] is unlikely, because the extent of photoaffinity labeling at 280 nm was low. The incorporation of radioactivity into albumin is dependent upon the duration of irradiation. A deviation from linearity is observed with increasing photolysis time because photocrosslinking and photodestruction of albumin occurs. This is indicated by an increasing amount of Coomassie blue staining material along the gel track lowering the amount of monomeric albumin. A linear relationship between labeling and duration of photolysis is obtained, if the different amounts of monomeric albumin on the gels after different photolysis times are considered or if the ratio of incorporated radioactivity/μg protein is plotted against photolysis time (Fig. 2B). The direct cross-linking of [^{3}H]benzylpenicillin to its binding proteins by ultraviolet irradiation has advantages compared to conventional photoaffinity labeling. The procedure is simple and circumvents synthetic procedures necessary for the introduction of photolabile groups. This eliminates the possibility that the specific interaction between the ligand and its receptor molecule may be disturbed by the chemical modification of the ligand.

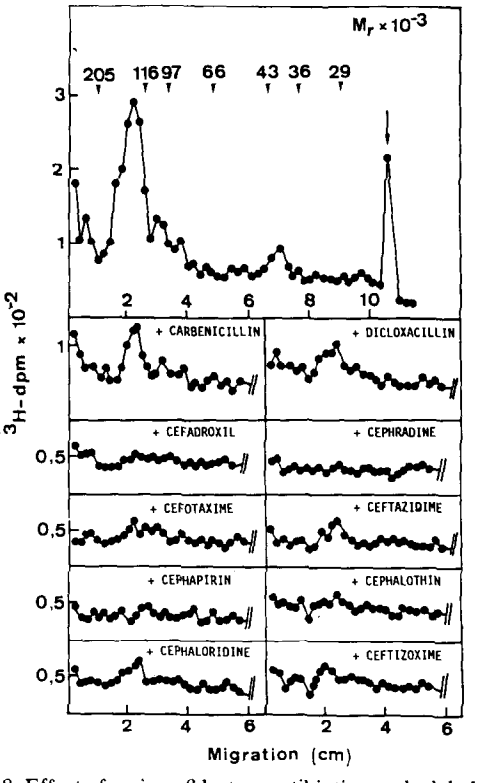

Fig. 8. Effect of various β-lactam antibiotics on the labeling of benzylpenicillin binding polypeptides from rabbit small intestine. 200 μg of brush border membrane vesicles from rabbit small intestine were photolabeled with 0.42 μM (3.1 μCi) [^{3}H]benzylpenicillin in the absence of inhibitors and in the presence of 250 μM of the indicated β-lactam antibiotics. Total acrylamide concentration was 7.5%. For all other conditions see legend to Fig. 5.

In order to identify protein components of the intestinal transport system for α-aminocephalosporins and dipeptides, brush border membrane vesicles from the small intestine of rabbit, rat and pig were photolabeled with [^{3}H]benzylpenicillin at 254 nm. Since the irradiation of membrane proteins with UV light may damage proteins, a compromise between the duration of photolysis and a sufficient extent of photoaffinity labeling has to be made. The H^+-dependent uptake of cephalexin into brush border membrane vesicles from rabbit small intestine was not significantly affected by irradiation at 254 nm up to 3 min (data not shown). Therefore, photoaffinity labeling of intestinal brush border membrane vesicles at 254 nm was performed for 2 min. Under these conditions a labeling of the intact dipeptide transporter should occur. A specific labeling of membrane polypeptides with apparent molecular weights of 127,000, 100,000 and 90,000 with highest labeling of the 127,000 molecular weight polypeptide was obtained (Fig. 3). The covalently attached benzylpenicillin could not be released from the proteins by treatment of the vesicles with hydroxylamine. This finding excludes the possibility that irradiation of the membrane vesicles in the presence of [^{3}H]benzylpenicillin led to an acylation of proteins like in the case of penicillin binding proteins from bacteria [26]. Compounds with peptide structure—dipeptides and β-lactam antibiotics—decreased the extent of labeling of the 127,000 molecular weight polypeptide indicating that these compounds also bind to this polypeptide. Other substrates like amino acids, bile salts or D-glucose, which are transported across the intestinal brush border membrane by carrier-mediated processes [18, 25, 27–29] had no influence on the labeling. With photolabile derivatives of cephalexin and glycyl-L-proline just recently a specific binding protein for dipeptides and cephalosporins of molecular weight 127,000 was identified in the brush border membrane from rat small intestine [17, 39]. The labeling of this cephalosporin and dipeptide binding polypeptide was decreased by orally active β-lactam antibiotics and dipeptides [39]. We therefore suggest that benzylpenicillin, cephalosporins and dipeptides interact with a common membrane protein of molecular weight 127,000 in the brush border membrane. Compounds which decreased the labeling of this 127,000 molecular weight polypeptide also inhibited the H^+-dependent uptake of cephalexin. β-Lactam antibiotics which are not absorbed from the gastrointestinal tract inhibited the uptake of orally active α-aminocephalosporins and also decreased the labeling of the 127,000 molecular weight polypeptide. From these results we conclude that all β-lactam antibiotics—penicillins as well as cephalosporins—bind to the intestinal uptake system for dipeptides and orally active α-aminocephalosporins, no matter whether the respective β-lactam antibiotic is absorbed or not. Hitherto unknown structural features of the β-lactam antibiotic molecule determine whether the β-lactam antibiotic bound to the dipeptide transporter from the luminal site is translocated across the intestinal brush border membrane or not.

REFERENCES

1. Dixon C and Mizen LW, Absorption of amino penicillins from everted rat intestine. *J Physiol (Lond.)* **269**: 549–559, 1977.
2. Quay JF and Foster L, Cephalexin penetration of the surviving rat intestine. *Physiologist* **13**: 287, 1970.
3. Quay JF, Transport interaction of glycine and cephalexin in rat jejunum. *Physiologist* **15**: 241, 1972.
4. Kimura T, Endo H, Yoshihawa M, Muranishi S and Sezaki K, Carrier-mediated transport systems for aminopenicillins in rat small intestine. *J Pharm Dyn* **1**: 262–267, 1978.
5. Tsuji A, Nakashima E, Kagami I and Yamana T, Intestinal absorption mechanism of amphoteric β-lactam antibiotics I: Comparative absorption and evidence for saturable transport of amino-β-lactam antibiotics by *in situ* rat small intestine. *J Pharm Sci* **70**: 768–772, 1981.
6. Miyazaki K, Ohtani K, Umeniwa K and Arita T, Intestinal absorption of several β-lactam antibiotics. III. Competitive inhibition behaviour among zwitterionic β-lactam antibiotics in the rat intestinal absorption. *J Pharm Dyn* **5**: 555–563, 1982.
7. Kimura T, Yamamoto T, Mizuno M, Suga Y, Kitade S and Sezaki H, Characterization of aminocephalosporin transport across rat small intestine. *J Pharm Dyn* **6**: 246–253, 1983.
8. Nakashima E, Tsuji A, Mizuo H and Yamana T, Kinetics and mechanism of *in vitro* uptake of amino-β-lactam antibiotics by rat small intestine and relation to the intact-peptide transport system. *Biochem Pharmacol* **33**: 3345–3352, 1984.
9. Okano T, Inui K, Maegawa H, Takano M and Hori R, H^+-coupled uphill transport of aminocephalosporins via the dipeptide transport system in rabbit intestinal brush-border membranes. *J Biol Chem* **261**: 14130–14134, 1986.
10. Okano T, Inui K, Takano M and Hori R, H^+-gradient-dependent transport of aminocephalosporins in rat intestinal brush-border membrane vesicles. *Biochem Pharmacol* **35**, 1781–1786, 1986.
11. Kimura T, Yamamoto T, Ishizuka R and Sezaki H, Transport of cefadroxil, an aminocephalosporin, across the small intestinal brush border membrane. *Biochem Pharmacol* **34**: 81–84, 1985.
12. Kwan KC and Rogers JD, Pharmacokinetics of β-lactam antibiotics. In: *Antibiotics Containing the Beta-Lactam Structure*, Part II (Eds. Demain AL and Solomon NA), pp. 247–370. Springer-Verlag, Berlin, 1983.
13. Penzotti SC and Poole JW, Transport mechanisms of β-lactam antibiotics across everted rat gut. *J Pharm Sci* **63**: 1803–1806, 1974.
14. Stewart HE and Jackson MJ, Transport of benzylpenicillin in gastrointestinal epithelia of the rat *in vitro*. *J Pharm Exp Ther* **218**: 453–458, 1981.
15. Tsuji A, Miyamato E, Kubo O and Yamana T, GI Absorption of β-lactam antibiotics III: Kinetic evidence for *in situ* absorption of ionized species of monobasic penicillins and cefazolin from the rat small intestine and structure-absorption rate relationships. *J Pharm Sci* **68**: 812–816, 1979.
16. Kramer W, Intestinal absorption of β-lactam antibiotics: Uptake and binding of benzylpenicillin by intestinal brush border membrane vesicles. *Naunyn-Schmiedeberg's Arch Pharmacol* **361**: R46, 1987.
17. Kramer W, Transport of β-lactam antibiotics by intestinal brush border membrane vesicles. *Biol Chem Hoppe-Seyler* **368**: 1261, 1987.
18. Burckhardt G, Kramer W, Kurz G and Wilson FA, Inhibition of bile salt transport in brush-border mem-

brane vesicles from rat small intestine by photoaffinity labeling. *J Biol Chem* **258**: 3618–3622, 1983.
19. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
20. Hopfer U, Nelson K, Perrotto J and Isselbacher KJ, Glucose transport in isolated brush border membranes from rat small intestine. *J Biol Chem* **248**: 25–32, 1973.
21. Berner W, Kinne R and Murer H, Phosphate transport into brush-border membrane vesicles isolated from rat small intestine. *Biochem J* **160**: 467–474, 1976.
22. Inui K-J, Okano T, Takano M, Kitazawa S and Hori R, Carrier-mediated transport of aminocephalosporins by brush border membrane vesicles isolated from rat kidney cortex. *Biochem Pharmacol* **32**: 621–626, 1983.
23. Gains N and Hauser H, Detergent-induced proteolysis of rabbit intestinal brush border vesicles. *Biochim. Biophys Acta* **646**: 211–217, 1981.
24. Wessel D and Flügge UJ, A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem* **138**: 141–143, 1984.
25. Kramer W, Burckhardt G, Wilson FA and Kurz G, Bile salt-binding polypeptides in brush-border membrane vesicles from rat small intestine revealed by photoaffinity labeling. *J Biol Chem* **258**: 3623–3628, 1983.
26. Spratt BG, Properties of the penicillin-binding proteins of *Escherichia coli* K 12. *Eur J Biochem* **72**: 341–352, 1977.
27. Hosang M, Gibbs EM, Diedrich DF and Semenza G, Photoaffinity labeling and identification of (a component of) the small intestinal Na^+, D-glucose transporter using 4-azidophlorizin. *FEBS Lett* **130**: 244–248, 1981.
28. Wright EM and Peerce BE, Identification and conformational changes of the intestinal proline carrier. *J Biol Chem* **259**: 14993–14996, 1984.
29. Peerce BE and Wright EM, Sodium-induced conformational changes in the glucose transporter of intestinal brush borders. *J Biol Chem* **259**: 14105–14112, 1984.
30. Tsuji A, Terasaki T, Tamai I, Nakashima E and Takanosu K, A carrier-mediated transport system for benzylpenicillin in isolated hepatocytes. *J Pharm Pharmacol* **37**: 55–57, 1985.
31. Tsuji A, Terasaki T, Takanosu K, Tamai I and Nakashima E, Uptake of benzylpenicillin, cefpiramide and cefazolin by freshly prepared hepatocytes. Evidence for a carrier-mediated transport system. *Biochem Pharmacol* **35**: 151–158, 1986.
32. Suzuki H, Sawada Y, Sugiyama Y, Iga T and Hanano M, Transport of benzylpenicillin by the rat choroid plexus *in vitro*. *J Pharm Exp Ther* **242**: 660–665, 1987.
33. Bayley H and Knowles JR, Photoaffinity labeling. *Methods Enzymol.* **46**: 69–114, 1977.
34. Bayley H, Photogenerated reagents in biochemistry and molecular biology. In: *Laboratory Reagents in Biochemistry and Molecular Biology* 12 (Eds. Work TS and Burdon RH). Elsevier, Amsterdam, 1983.
35. Chowdhry V and Westheimer FH, Photoaffinity labeling of biological systems. *Annu Rev Biochem* **48**: 293–325, 1979.
36. Deziel M, Pegg W, Mack E, Rothstein A and Klip A, Labelling of the human erythrocyte glucose transporter with ^{3}H-labelled cytochalasin B occurs via protein photoactivation. *Biochim Biophys Acta* **772**: 403–406, 1984.
37. Klip A, Walker D, Ransome KJ, Schroer DW and Lienhard GE, Identification of the glucose transporter in rat skeletal muscle. *Arch Biochem Biophys* **226**: 198–205, 1983.
38. Graham D, Pfeiffer F and Betz H, Photoaffinity-labelling of the glycine receptor of rat spinal cord. *Eur J Biochem* **131**: 519–525, 1983.
39. Kramer W, Identification of identical binding polypeptides for cephalosporins and dipeptides in intestinal brush-border membrane vesicles by photoaffinity labeling. *Biochim Biophys Acta* **905**: 65–74, 1987.