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Effects of Trypsin and Protein Modification on the Renal Transporter of *p*-Aminohippurate

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Summary. Basal-lateral membranous vesicles prepared from rabbit renal cortex exhibited Mg^{2+}-stimulated, probenecid-inhibitable transport of *p*-aminohippurate (PAH). This uptake could be completely eliminated by incubating the membranes with trypsin at a weight ratio of 1 : 700 (trypsin/membrane protein). The loss of PAH uptake activity occurred in two stages. Over the first ten minutes of the vesicles' exposure to trypsin, there was a nearly linear loss, with respect to time, of about 80% of the PAH uptake activity. The remaining 20% of activity was resistant to further trypsin digestion for the next ten minutes, but by twenty-five minutes a total inactivation of the uptake activity occurred. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of normal and trypsin-treated vesicles showed very little degradation of proteins. However, two minor polypeptides (M_r - 410,000 and 388,000) were degraded during the first ten minutes of the membranes' exposure to trypsin. After twenty minutes of exposure, two other poypeptides (M_r = 94,500 and 87,500) were degraded. Chymotrypsin and clostripain also caused a loss of PAH transport activity. However, compared to the effects of trypsin, the effects of these two proteases were less complete, slower in onset, and for clostripain, a much higher concentration of enzyme was required. Other functions or properties of the vesicles including morphological appearance, degree of vesiculation, glucose space or Na^+-dependent L-glutamate transport and Na^+, K^+-ATPase activity were not altered by the concentration of trypsin which abolished 80% of the transport of PAH. Thus, it is possible that one or more of the degraded polypeptides detected by polyacrylamide gel electrophoresis comprises the PAH transporter. Furthermore, modification of the vesicles with phenylglyoxal led to a 38% loss of PAH uptake activity. This suggests that arginine residues may play an important role in the transport system.

Key Words trypsin · *p*-aminohippurate · basal-lateral membranes · vesicles · transport · kidney

Introduction

During the last decade, basal-lateral membranous vesicles have been successfully isolated from renal cortices of rat (Heidrich et al., 1972; Ebel, Aulbert & Merker, 1976; Inui et al., 1981; Scalera et al., 1981), dog (Kinsella et al., 1979*a*) and rabbit (Liang & Sacktor, 1977; Mamelok et al., 1981, 1982). These vesicles were shown to contain a specific transport system for *p*-aminohippurate (PAH) (Berner & Kinne, 1976; Kinsella et al., 1979*b*; Mamelok et al., 1982), capable of transporting PAH into the intravesicular space. The transport is inhibited by probenecid and it is enhanced by a Na^+ gradient in some species (Berner & Kinne, 1976; Kinsella et al., 1979*b*; Sheikh & Møller, 1982). Recently, studies from our laboratory indicated that the rate of uptake of PAH was enhanced by divalent cations, Mg^{2+} and Mn^{2+} being the most stimulatory (Tse et al., 1983). However, the basic structure and molecular properties of the PAH transporter are not well understood. Moreover, the surface topography of this transport system remains unknown.

Previous reports indicate that the PAH transport system is comprised primarily of proteins (Berner & Kinne, 1976; Holohan et al., 1979; Tse et al., 1982). Because the PAH transporter must translocate PAH from one side of the plasma membrane to the other it is almost certain that a portion of the transporter is located near the surfaces of the basal-lateral membrane. Proteolytic enzymes have been used as nonpenetrating probes to study proteins on the surface of membranes (Jain, 1973; Noonan, 1978). These proteases have been used successfully to elucidate the topological distribution and organization of proteins and glycoproteins located in intact plasma membranes (Knauf, 1979). Proteases can be very helpful in defining domains of membrane-bound proteins which are essential for either normal function of the membrane or for maintaining the stability of the membrane's structure (Cuatrecasas, 1974).

In this report we describe the effects, on PAH transport into renal basal-lateral membranous vesicles, of trypsin and of reagents known to react with

free amino groups of lysine residues or arginine residues. Trypsin was used because of its high specific activity and narrow substrate specificity. The results indicate that the transporter of PAH contains peptide bonds involving lysine and/or arginine residues which are exposed on the surface of the membrane. The cleavage of these peptide bonds by trypsin causes an irreversible loss of PAH uptake activity. The effect of tryptic digestion on the membrane's proteins was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Materials and Methods

Preparation of Membranes

Vesicles composed of basal-lateral membranes were prepared from rabbit renal cortices by a combination of differential centrifugation and sucrose gradient centrifugation in a zonal rotor as described previously (Mamelok et al., 1981, 1982). Only one change was made. In order to inhibit proteolysis, leupeptin (3.8 μM), an inhibitor for all trypsin-like proteases, was added to the buffer used for perfusing the kidney and homogenizing the cortex. The final pellet of basal-lateral membranes was suspended in buffer containing 50 mM mannitol and 6.9 mM Tris-HCl, pH 7.0. The concentration of protein in the suspension of membranes was between 8 and 13 mg/ml. The vesicles were kept at 4°C. Compared to a homogenate of renal cortex these basal-lateral membranes are enriched about 12-fold as determined by the increase in the specific activity of Na, K-ATPase (Mamelok et al., 1982).

Proteolytic Digestions

Aliquots of vesicles were incubated at 25°C with trypsin at a ratio of 700 μg membrane protein to 1 μg of trypsin. At different times, the proteolytic degradation was stopped by the addition of leupeptin, an irreversible inhibitor of trypsin (Umezawa & Aoyagi, 1977). Leupeptin was added in a ratio to trypsin of 1 (wt/wt). The digested vesicles were used immediately for uptake studies. With chymotrypsin, an incubation identical to that for trypsin was carried out except that the digestion was stopped by the addition of eight times the weight of turkey egg white trypsin inhibitor (Lineweaver & Murray, 1947), which inhibits chymotrypsin. Clostripain (5 mg/ml, final concentration) (E.C. 3.4.22.8) from clostridium histolyticum was activated by incubation at 25°C for 2 hr in 1 mM calcium acetate and 2.5 mM dithiothreitol just prior to use. About 900 ml of vesicles were incubated with activated clostripain at 25°C in a buffer containing 7.1 mM sodium phosphate, pH 7.6, and 0.51 mM dithiothreitol. The weight ratio of membrane vesicles to clostripain was 50 to 1. At appropriate intervals of time, the activity of clostripain was terminated with leupeptin in a manner similar to that of trypsin.

Transport Studies

Transport of PAH was measured by the rapid filtration method as previously described (Berner & Kinne, 1976; Mamelok et al., 1982). Briefly, the transport was carried out at 37°C in the presence of 0.1 mM PAH with or without 6.7 mM probenecid and an initial gradient of 5 mM Mg^{2+} except for the experiment depicted in Fig. 1 where 1 mM PAH was used. Probenecid-inhibitable uptake is determined by subtracting uptake in the presence of probenecid from uptake in the absence of probenecid. 6.7 mM probenecid produces maximal inhibition (Tse et al., 1983). Uptake of D-glucose was measured as previously reported (Hittelman, Mamelok & Prusiner, 1978, Mamelok et al., 1982) at 25°C in the presence of an initial 70 mM Na^+ gradient. The final concentration of D-glucose in the reaction mixture was 0.47 mM. The sodium was used as part of a routine reaction mixture, although we have shown that these basal-lateral membranes do not contain Na^+-dependent glucose transport (Mamelok et al., 1982). Uptake of glutamate with either a 100 mM gradient of Na^+ or K^+ was carried out at 30 sec, 1 min, 2 min and 60 min as described by Sacktor et al. (1981). Na^+-dependent uptake was determined by subtracting the uptake of L-glutamate in the presence of an initial gradient of 100 mM K^+ from the uptake in the presence of an initial gradient of 100 mM Na^+ (Sacktor et al., 1981). The exact conditions for each experiment are listed under the appropriate Figure.

Modification of Basal-Lateral Membrane Proteins

Basal-lateral membrane vesicles were modified with phenylglyoxal, a reagent relatively specific for arginine (Takahashi, 1968), and with two amino-specific reagents, citraconic anhydride (Dixon & Perham, 1968), and trinitrobenzenesulfonic acid (TNBS) (Okuyama & Satake, 1960; Satake et al., 1960). Since both phenylglyoxal and TNBS reacted with a free amino group of Tris, the vesicles used for these modifications were resuspended in 50 mM mannitol), 6.9 mM HEPES, pH 7.0 (buffer E). The modification mixture contained equal volumes of vesicles (18 to 26 mg/ml protein, of 30 mM phenylglyoxal, 30 mM citraconic anhydride or 6 mM TNBS buffered in 0.5 M ethylmorpholine, pH 8.0. The control contained only 0.5 M ethylmorpholine, pH 8.0. These reagents react with arginine and/or amino groups rapidly (within 5 to 10 min) (Dixon & Perham, 1968; Takahashi, 1968; Fields, 1972); thus, an incubation period of 30 min at 37°C was used to ensure adequate modification. After incubation the vesicles were washed twice by diluting them ten-fold in buffer E. The vesicles were separated from excess reagents by centrifugation at 13,000 rpm (SS 34 rotor, Sorvall) for 30 min. The final pellet was resuspended in buffer A to a final concentration of protein of 10 to 15 mg/ml. The vesicles were then used immediately for uptake studies.

Gel Electrophoresis

The proteins of the basal-lateral membrane before and after digestion by trypsin were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis by a modification of the method of Laemmli (1970). The separation gel contained 7.5% acrylamide and 0.18% bisacrylamide; the stacking gel contained 5% acrylamide and 0.11% bisacrylamide. No sodium dodecyl sulfate was present in either gel which resulted in similar and sometimes better resolution of the polypeptides compared to when SDS was present. The running buffer contained 0.19 M glycine, 0.033 M Tris, pH 8.3, 0.05% (wt/vol) sodium dodecyl sulfate and 0.011 M β-mercaptoethanol. About 150 μg of the denatured proteins were

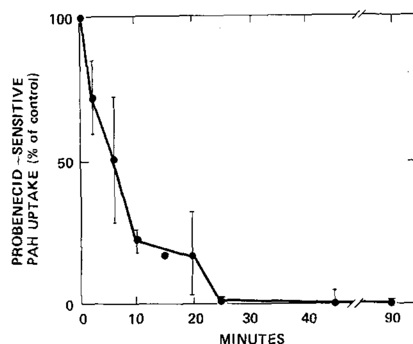

Fig. 1. The effect of digestion by trypsin, at various incubation times, on the probenecid-sensitive PAH uptake in basal-lateral membraneous vesicles. Each point is the average of three experiments (±SD). The uptakes were carried out with 1 mM PAH with and without 6.7 mM probenecid, for 10 sec at 37°C with a gradient of 5 mM Mg^{2+}. Probenecid-sensitive uptake is defined as the difference in uptake measured in the absence and presence of probenecid

Table 1. Effect of preincubation with PAH and probenecid on the trypsin inactivation of probenecid-sensitive uptake in basal-lateral membranous vesicles

Reagent used for preincubation[a]	Trypsin digestion	% of uptake[b]
Control buffer	No	100
	Yes	40
5 mM PAH	No	118
	Yes	19
6.7 mM probenecid	No	91
	Yes	36

[a] The vesicles were preincubated for 30 min at 37°C with either buffer alone (50 mM mannitol, 6.9 mM Tris-HCl, pH 7.0) or with buffer containing 5 mM PAH or 6.7 mM probenecid. About half of the vesicles were then incubated with trypsin for 10 min.
[b] 100% = 31 pmol/mg/min.

electrophoresed at 16°C with a constant current of 20 mA per slab gel until the tracking dye, 0.05% (wt/vol) bromophenol blue, passed the interface between the stacking and separation gel. The current was then increased to 30 mA. The gels were fixed and stained with Coomassie Blue as described by Weber and Osborn (1969). The gels were destained by several changes of a solution containing 7% (vol/vol) acetic acid and 10% (vol/vol) isopropanol. Two sets of markers of molecular weight were used: set A contained cross-linked bovine serum albumin (Sigma Chemicals) (M_r = 66,000, 132,000, 198,000 and 264,000) and set B contained thyroglobulin (subunit = 330,000), phosphorylase *a* (subunit = 93,000), bovine serum albumin (66,000) and catalase (subunit = 60,000).

Analytical and Enzymatic Assays

Na,K-ATPase was measured colorimetrically as previously described (Mamelok et al., 1982). Protein concentration was determined by the method of Bradford (1976).

Materials

All chemicals were of reagent grade. D[1-^{3}H(N)]-glucose, (15 Ci/mmol) was obtained from New England Nuclear. *p*-Amino(^{3}H)hippuric acid (374 mCi/mmol) was purchased from Amersham/Searle. L-(3,4-^{3}H)glutamic acid (35 Ci/mmol) was obtained from ICN Pharmaceuticals.

Results

The first protease used for the present studies was trypsin. This enzyme cleaves only peptide bonds involving the carboxyl groups of either arginine or lysine residues. A low level of trypsin, at a ratio of 1 μg trypsin to 700 μg of membrane proteins (0.14% wt/wt), was used for the experiments. Basal-lateral membranous vesicles were incubated with trypsin in order to determine the time course of the destruction of probenecid-sensitive PAH uptake. The results, averaged from three experiments, are shown in Fig. 1. During the first 10 min of the vesicles' exposure to trypsin, there was a nearly linear loss of about 80% of the uptake activity. The remaining uptake activity was relatively resistant to further degradation by trypsin for another 10 min. Finally, a total inactivation of the probenecid-sensitive PAH uptake was observed after 25-min incubation. Preincubation of trypsin with leupeptin completely eliminated the inactivation of transport by trypsin.

Because of its size, trypsin would only cleave peptide bonds on the surface of the vesicle. We asked whether the presence of a substrate or of an inhibitor of the transport system might cause a conformational change of the transporter which, in turn, would result in removing the susceptible peptide bonds from the membrane's surface. Thus, we determined whether PAH or probenecid could protect the transporter from digestion. The incubation procedure was as follows: vesicles were incubated with either 5 mM PAH or 6.7 mM probenecid at 37°C for 30 min. Trypsin was then added to half the vesicles, and the mixture was incubated for 10 min at 25°C. Then leupeptin was added to stop proteolysis. The trypsin-treated and untreated vesicles were washed twice with buffer by centrifugation at 13,000 rpm (SS-34 rotor, Sorvall) for 30 min. The final pellet was resuspended in buffer for transport experiments. The results are listed in Table 1. The uptake of PAH into the vesicles, in the absence of trypsin,

Table 2. Probenecid-insensitive uptake

	6-min incubation			10-min incubation		
	Trypsin (pmol/mg/min)	Trypsin + leupeptin	Uptake in presence of trypsin ÷ uptake in presence of trypsin + leupeptin	Trypsin (pmol/mg/min)	Trypsin + leupeptin	Uptake in presence of trypsin ÷ uptake in presence of trypsin + leupeptin
Exp. 1	6.1 ± 1.6	6.1 ± 1.8	1.00	4.8 ± 1.4	5.4 ± 1.2	0.89
Exp. 2	2.5 ± 0.9	3.9 ± 0.6	0.64	3.5 ± 0.9	2.8 ± 0.2	1.25
Exp. 3	10.5 ± 2.1	10.4 ± 3.1	1.01	11.9 ± 1.5	12.2 ± 2.6	0.97

occurred at a rate of 31 pmol/mg protein at 1 min. When neither PAH nor probenecid was present in the preincubation of the vesicles, a 10-min exposure of the vesicles to trypsin caused a 60% loss of uptake activity at 1 min. When PAH or probenecid was present there was, respectively, an 81 and 64% loss of uptake activity.

To determine if trypsin caused an increased leakiness of vesicles to PAH we studied the effect of trypsin on uptake which was not inhibited by probenecid. The ratio of probenecid-insensitive PAH uptake, measured at 1 min in the presence of 6.7 mM probenecid, was measured in vesicles incubated with trypsin or with inactivated trypsin (trypsin plus leupeptin). These experiments were performed in vesicles exposed to active or inactive trypsin for 6 and 10 min. The ratio of the probenecid-insensitive uptake after incubation of vesicles with trypsin to the probenecid-insensitive uptake after incubation with inactive trypsin was 0.88 ± 0.12 (±SE, $n = 3$) and 1.04 ± 0.11 (±SE, $n = 3$) after 6- and 10-min incubation, respectively. The numerical values for uptake in three such experiments are listed in Table 2. Trypsin did not cause an increase in the probenecid-insensitive uptake, indicating that there was no increase in a nonspecific leakiness of the vesicles to PAH.

It was possible that the inactivation of PAH transport by trypsin was the result either of a general change of the membrane's permeability due to the proteolysis of the membrane's proteins (Jain, 1973) or of the destruction of closed vesicles. Therefore, experiments were carried out to investigate these possibilities. The uptake of D-glucose was studied. The results of a typical experiment, seen in Fig. 2, show that the uptake of glucose after digestion by trypsin was about twofold higher when measured at 30 sec and about 16% higher at 1 min, when compared to uptake of glucose in the absence of tryptic digestion. This suggests that the vesicles may have become leaky to glucose after exposure to trypsin. However, the uptake of glucose into digested and control vesicles at 60 min was not altered indicating that the total intravesicular spaces of the trypsin-treated and untreated vesicles were the same. Thus, destruction of vesicles did not seem to occur. Furthermore, in order to determine directly whether trypsin altered the morphology of the vesicles, electron micrographs of vesicles treated with trypsin for 10 min were obtained. There were no changes in the degree of vesiculation nor in the apparent diameter of the vesicles (*data not shown*).

It has been shown that tryptic digestion of erythrocytes causes an increase in the density of surface charge (Seaman & Uhlenbruck, 1962). Therefore, the decrease of PAH uptake caused by trypsin could have resulted from changes of the membrane's net charge, rather than from a direct effect on the PAH transporter. In order to distinguish these two effects, the uptake into the vesicles of another organic anion, glutamate, was studied. There was no change in the sodium-dependent nor in the sodium-independent glutamate uptake in control and trypsin-treated vesicles (Fig. 3).

The enzymatic activity of membrane-bound Na,K-ATPase, a marker enzyme for the basal-lateral membrane, was also determined before and after 10-min incubation with trypsin. The specific activity measured before and after trypsin digestion was 767 ± 203 (±SD, $n = 3$) and 808 ± 338 (±SD, $n = 3$) nmol phosphate produced/mg protein/min, respectively.

Since the probenecid-sensitive PAH uptake could be inactivated by trypsin, it seemed likely that there was degradation of a transport protein. Thus, we attempted to determine if degradation of a specific protein could be observed. The vesicles were digested with trypsin as described above. At the time intervals depicted in Fig. 1, part of the vesicles were removed to a test-tube containing enough leupeptin to inactivate the trypsin completely. Immediately, the proteins of the vesicles were solubilized and denatured. The proteins were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The results are shown in Fig. 4.

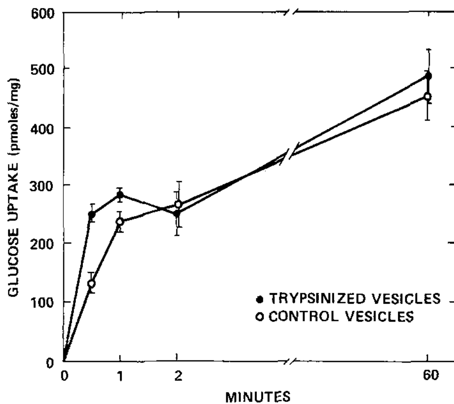

Fig. 2. D-glucose uptake in basal-lateral membranous vesicles. Vesicles exposed to trypsin for 10 min (●—●) and control vesicles (O—O). Standard errors of the mean are indicated

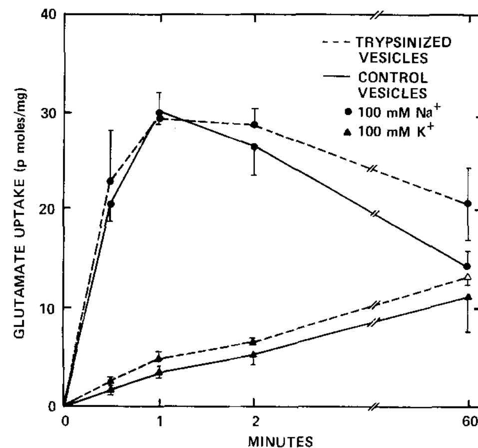

Fig. 3. L-glutamate uptake in basal-lateral membranous vesicles. Vesicles exposed to trypsin for 10 min (dashed lines) and control vesicles (solid lines). Initial gradient of 100 mM Na^+ (●—●) or 100 mM K^+ (▲—▲). Standard errors of the mean are indicated

The protein profiles of the vesicles, even after 90 min of incubation with trypsin, remained relatively unchanged. This indicates very little proteolysis. This low level of degradation was confirmed by determining the trichloroacetic acid soluble free amino groups. None could be detected. However, two polypeptides, I and II (M_r = 410,000 and 388,000), were degraded during the first 10 min of incubation with trypsin concurrent with the loss of 80% of probenecid-sensitive uptake. Two other polypeptides, III and IV (M_r = 94,500 and 87,500), were lost after 20-min digestion, concommitant with the total loss of uptake activity towards PAH.

The effects of two other proteases, chymotrypsin and clostripain, on the probenecid-sensitive transport of PAH into the vesicles were also determined (Fig. 5). During the first 20 min of incubation with chymotrypsin, there was a small (about 10 to 20%) decrease in uptake activity. With a longer period of incubation (45 min), there was a 50% loss of activity. Similarly there was a 50% decrease of uptake activity with clostripain after a 15-min incubation, and there was no further decrease, even after 60 min of incubation. Interestingly, during the first 10 min of clostripain incubation, there was a slight increase in uptake activity. In contrast, trypsin inactivated about 80% of the uptake activity during an incubation of 10 min.

Because trypsin hydrolyzes peptide bonds involving arginine or lysine residues, the destruction of PAH transport by trypsin led us to conclude that the transporter contains a protein that has an arginine and/or lysine peptide bond exposed at the surface of the membrane and that this bond was essential for maintaining the functional integrity of the transporter. In order to determine whether these cationic moieties might play a role in the function of

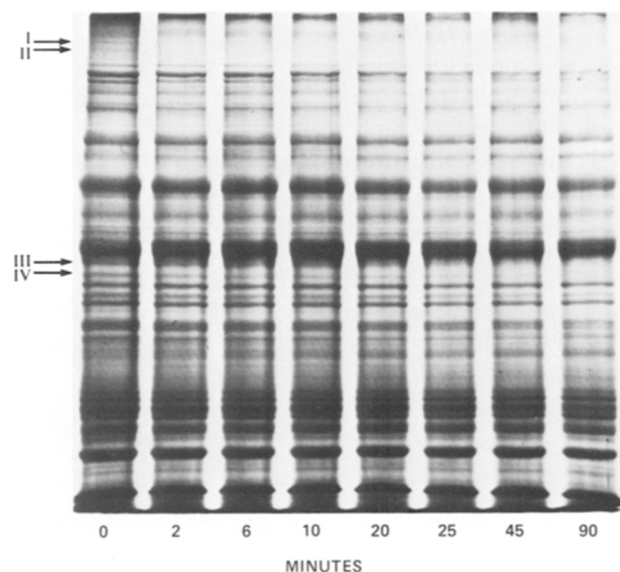

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of basal-lateral membranous vesicles. Electrophoresis of 150 mg of membrane protein was performed in a 7.5% acrylamide gel after various times of incubating vesicles with trypsin. The molecular weights of the polypeptides indicated in the gel are estimated to be: I = 410,000; II = 388,000; III = 94,500; IV = 87,500

the transporter, we examined the effect, on probenecid-inhibitable uptake, of reagents which react with arginine and amino groups. The results of these experiments are shown in Table 3. Phenylglyoxal, which reacts relatively specifically with arginine residues, consistently inhibited the transport of PAH into basal-lateral membranous vesicles. TNBS

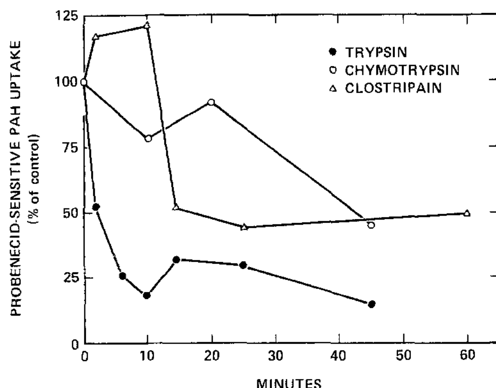

Fig. 5. Effect of proteases on probenecid-sensitive PAH uptake in basal-lateral membranous vesicles. The vesicles were incubated with either trypsin (●—●), chymotrypsin (○—○) or clostripain (△—△) for the times indicated. The PAH uptake was carried out for 1 min with 0.1 mM PAH. The results with trypsin are means determined from four experiments and those for chymotrypsin and clostripain are means from two experiments

which reacts with ε-amino groups of lysine residues also inhibited transport. Another reagent, citraconic anhydride, which reacts with ε-amino groups of lysine residues, did not consistently inhibit the transport of PAH. Transport activity towards PAH was on average reduced to 62% of control by phenylglyoxal and to 71% of control by TNBS. The data was evaluated by a paired Student's t-test. The inhibition by phenylglyoxal was significant, $0.005 < P < 0.01$ and the inhibition by TNBS was significant, $0.02 < P < 0.05$.

Discussion

The aim of the present work was to determine the stability of the PAH transport system of renal basal-lateral membranes towards proteolytic degradation. The probenecid-sensitive PAH uptake was rapidly inactivated by treating membranous vesicles with trypsin; there was a loss of 80% of transport activity within 10 min. The weight ratio of trypsin-to-membrane proteins used for the present work was 1 : 700. This ratio is much lower than those reported to degrade other membrane-bound binding proteins or transport systems (Glossmann & Neville, 1972; Wang, Gurd & Mahler, 1975; Hsu et al., 1982). Thus, the PAH uptake system was very sensitive to trypsin. This is certainly the result of the proteolytic action of this enzyme since the trypsin inhibitor, leupeptin, could prevent inactivation of the uptake activity by trypsin and because the addition of a nonenzymatic protein such as bovine serum albumin had no inhibitory effects. Trypsin, which hydrolyzes only peptide bonds containing either arginine or lysine residues, has a very narrow substrate specificity. This implies that the PAH transport system contains arginine or lysine peptide bonds near the membrane's surface where they are accessible to trypsin. Since neither PAH nor probenecid could protect the PAH transporter from inactivation by trypsin, it seems that the sites of cleavage by trypsin are not removed from the surface when the transporter interacts with substrates. The decreased uptake did not seem to be due to increased nonspecific leakiness of the vesicles towards PAH since uptake not inhibited by probenecid was unaltered by trypsin.

The results from the present studies suggest that arginine residues of the transport proteins play a more important role than lysine residues in the PAH transporter. This conclusion is supported by the results of several studies. Clostripain, which has an even narrower substrate specificity than trypsin, was capable of destroying transport activity toward PAH. Clostripain is an endopeptidase which predominantly cleaves peptide bonds involving carboxyl groups of arginine (Mitchell & Harrington, 1971). Thus, an arginine residue itself or a section of a protein containing a peptide bond of arginine is essential for maintaining a totally intact transporter. The results obtained from experiments with phenylglyoxal strengthen the conclusion that a residue of arginine is important to the integrity of the transporter. Under the conditions used in our studies phenylglyoxal has been shown to react mainly with guanido groups of arginine and possibly with N-terminal amino groups (Takahashi, 1968). Furthermore, citraconic anhydride which reacts selectively with lysine and N-terminal amino groups did not inhibit transport. This suggests that the involvement of N-terminal amino groups in the uptake of PAH is highly unlikely. It cannot be determined from the present data whether the arginine residues modified by phenylglyoxal and the arginine residues of the peptide bonds cleaved by trypsin and clostripain were identical. Further work is required to clarify this matter.

That lysine is not important to the transporter cannot be ruled out by these studies. First, trypsin was able to completely abolish probenecid-sensitive transport whereas the maximal effect obtained with clostripain was an inhibition of 50%. This difference could be due either to the slightly broader range of peptide bonds susceptible to cleavage by trypsin or to a greater accessibility of susceptible bonds to trypsin than to clostripain.

Another suggestion that lysine may be involved

Table 3. Effects of group specific reagents on probenecid-inhibitable PAH uptake

Exp.	Control[a]	Citraconic anhydride		Phenylglyoxal		TNBS	
	Uptake (pmol/mg/min)	Uptake (pmol/mg/min)	% Activity	Uptake (pmol/mg/min)	% Activity	Uptake (pmol/mg/min)	% Activity
1	35.4	29.3	83	18.9	53	25.4	72
2	23.6	33.8	143	—	—	21.5	91
3	44.2	31.9	72	18.3	41	22.8	52
4	28.3	24.3	86	27.6	98	—	—
5	15.6	9.0	58	6.1	39	8.1	52
6	24.1	27.7	115	20.3	84	22.8	95
7	33.5	56.1	167	19.4	57	—	—
8	21.2	—	—	—	—	14.0	66
Mean ± SD% activity:							
	100		103 ± 40		62 ± 24[b]		71 ± 19[c]

[a] No reagent added.
[b] $P < 0.01$ by two-tailed, paired Student's t test.
[c] $P < 0.05$ by two-tailed, paired Student's t test.

comes from the studies with TNBS, which reacts with lysine and with N-terminal amino groups, and which inhibited transport of PAH. However, TNBS also can react with sulfhydryl groups (Kotaki, Harada & Yagi, 1964; Fields, 1972). We have recently shown that reagents which react with sulfhydryl groups can inhibit the transport of PAH across basal-lateral membranes (Tse et al., 1983; Tse et al., *in press*). Some preliminary data suggest that the action of TNBS may be through reactions with sulfhydryl groups. When the membranes and TNBS were incubated for 15 instead of 30 min at either pH 7.0 or pH 8.8, the PAH uptake activity was not significantly altered by TNBS (*unpublished observations*). The reaction of TNBS with sulfhydryl groups proceeds at a much slower rate than the reaction of TNBS with amino groups (Kotaki et al., 1969). Also few if any sulfhydryl groups will react with TNBS until all the available amino groups are reacted (Kotaki et al., 1969). Thus, the 30 min of incubation of membranes with TNBS which are required to produce inhibition of transport suggest that the slower reaction with sulfhydryl groups may be responsible for inhibition of transport. Other possible situations could also explain the inhibition of PAH transport by TNBS and the lack of inhibition by citraconic anhydride. It is possible that the two compounds are able to gain access to or to react with a difference set of amino groups in the membranes. Also, the reaction of TNBS with phosphatidylethanolamine may contribute to its effect. Also, TNBS could alter the membrane in other ways, possibly through hydrophobic interactions with the membrane (Plapp, Moore & Stein, 1971). A final possibility is that the longer incubation time necessary for TNBS to inhibit PAH uptake is related to a slower rate of penetration to its site of action. Further work is required to determine why TNBS is inhibitory and citraconic anhydride is not.

The data indicate that 10-min digestion by a low concentration of trypsin has rather limited effects on the membrane's integrity. This conclusion is based on several observations. First of all, vesicles exposed to trpsin retain their general structure since electron microscopy reveals neither a change in the vesicles' size nor in the degree of vesiculation. Also the "glucose space" at equilibrium remained unchanged. The Na^+-dependent uptake into vesicles of another anionic substance, L-glutamate, was not affected by low concentrations of trypsin. The Na^+-independent uptake of glutamate, probably representing diffusion, was minimally increased by trypsin. Thus, although the vesicles might have become slightly leaky to glutamate, there was no evidence for an increased negativity in the membrane's charge density. In contrast, the uptake of a neutral substrate, D-glucose, was increased by trypsin, indicating a possible increased leakiness to glucose. Also, there was no change in the activity of Na,K-ATPase, which, in a purified form has been shown to be degraded by trypsin at the higher weight ratios of 1 : 40 to 1 : 10 (Jorgensen, 1975; Churchill & Hokin, 1976). Most importantly, there was no evidence that trypsin decreased probenecid-sensitive PAH uptake by increasing a leak of PAH by a probenecid-insensitive pathway (Table 2).

We have shown that the basal-lateral membranes used in these experiments exhibit both pro-

benecid-inhibitable uptake of PAH into an osmotically active space, and also probenecid-inhibitable binding (Mamelok et al., 1982). Since trypsin can completely abolish all measurable probenecid-inhibitable interactions, it must be degrading a specific binder and a specific transporter.

As observed in a representative Coomassie Blue stained gel (Fig. 4), very few proteins were degraded during the course of a tryptic digestion. Even with periods of incubation of 90 min, at which time the PAH uptake activity was completely inactivated, there was hardly any detectable degradation of the membrane's proteins. This indicates that the proteins involved in the PAH transport system comprise a very small fraction of the total protein. Furthermore, no peptide fragments released from vesicles treated with trypsin could be detected. These data emphasize the limited effects of low concentrations of trypsin on basal-lateral membranes. Nevertheless, several minor polypeptides were degraded during the course of the treatment with trypsin. The loss of polypeptides I and II during the first 10 min of tryptic digestion as well as the loss of polypeptides III and IV after 25 to 60 min of digestion occur in parallel with the loss of PAH uptake activity depicted in Fig. 1. It is possible that only one or a combination of these four polypeptides are somehow related to the PAH transport system. However, further investigations are required to implicate these four polypeptides in the transport of PAH in basal-lateral membranes.

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