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Na^+ AND H^+ GRADIENT-DEPENDENT TRANSPORT OF *p*-AMINOHIPPURATE IN MEMBRANE VESICLES FROM DOG KIDNEY CORTEX

FRANS G. M. RUSSEL*†, PETER E. M. VAN DER LINDEN*, WIM G. VERMEULEN*, MARC HEIJN*, CAREL H. VAN OS‡ and CEES A. M. VAN GINNEKEN*

*Department of Phamacology and ‡Department of Physiology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

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Abstract—The transport of *p*-aminohippurate (PAH) was studied in basolateral (BLMV) and brush border membrane vesicles (BBMV) isolated from dog kidney cortex. Imposition of an inwardly directed 100 mN Na^+ gradient stimulated the uptake of 50 μM [^{3}H]PAH into BLMV, whereas a pH gradient (pH_{out} = 6.0, pH_{in} = 7.4) only slightly enhanced uptake. The Na^+ gradient-dependent uptake of PAH was electroneutral, saturable and sensitive to inhibition by probenecid and several anionic drugs, with (apparent) $K_m = 0.79 \pm 0.16$ mM, $V_{max} = 0.80 \pm 0.05$ nmol/mg protein,15 sec and K_i for probenecid = 0.08 ± 0.01 mM. Simultaneous imposition of the pH gradient (outward OH^- gradient) and inward Na^+ gradient stimulated PAH uptake significantly over that with an Na^+ gradient alone. These results are consistent with an Na^+ gradient-stimulated PAH/OH^- exchange mechanism in the basolateral membrane. In BBMV, PAH uptake could be stimulated by an outwardly directed OH^- gradient as well as an inward Na^+ gradient. Both gradients could drive PAH transport via a mediated probenecid-sensitive pathway. Na^+ gradient-stimulated uptake was electrogenic with a (apparent) $K_m = 4.93 \pm 0.57$ mM, $V_{max} = 6.71 \pm 0.36$ nmol/mg protein,15 sec and $K_{i,prob} = 0.13 \pm 0.01$ mM. The kinetic parameters for PAH/OH^- exchange were virtually the same, (apparent) $K_m = 5.72 \pm 0.49$ mM, $V_{max} = 7.87 \pm 0.33$ nmol/mg protein,15 sec and $K_{i,prob} = 0.16 \pm 0.02$ mM. When both the Na^+ and pH (outward OH^-) gradient were simultaneously imposed an almost twofold stimulation in uptake was observed over that with either an Na^+ or pH gradient alone. These results suggested that both gradients stimulate PAH transport in BBMV via the same pathway. However, inhibition experiments with various organic anions showed that the specificities of Na^+ and pH gradient-stimulated PAH uptake do not entirely overlap. Thus, our results support a simple transport in BBMV, but it cannot be excluded that two separate pathways are involved.

Most of our present knowledge about the renal handling of organic anionic drugs comes from studies with *p*-aminohippurate (PAH). In the mammalian kidney transport of PAH and other organic anions is confined to the proximal tubule. PAH is actively secreted by the tubular cells into the urine against an electrochemical gradient. The active step in the transepithelial transport appears to be the uptake across the basolateral membrane into the cells, since PAH is accumulated to much higher concentrations in the cells than in the extracellular fluid. The high intracellular concentration is probably the main driving force for the mediated movement across the brush border membrane into the lumen [1,2].

In the past ten years the use of isolated membrane vesicles has contributed to a better understanding of the role that each plasma membrane plays in the transcellular transport of PAH. However, despite numerous vesicle studies there is no general agreement on the mechanisms and the driving forces that govern PAH transport across the basolateral and brush border membrane.

Several investigators have demonstrated mediated transport of PAH into basolateral membrane vesicles (BLMV) from rat [3–6], rabbit [6–8] and dog [9], which was stimulated by a Na^+ gradient and sensitive to probenecid inhibition. However, only Sheikh and Møller [7] were able to achieve uptake values above equilibrium, using BLMV from rabbit kidney. On the other hand Tse *et al.* [10] observed just a minor stimulating effect of Na^+ in rabbit BLMV, while Mg^{2+} and other divalent cations significantly enhanced probenecid-sensitive PAH transport. Berner and Kinne [3] suggested a nonspecific effect of Na^+ by creating an inside positive diffusion potential which stimulated PAH anion uptake, while others observed only a small [4] or no effect [5,7] of the membrane potential on Na^+-dependent PAH uptake. Taking these results together most studies point to a direct coupling of Na^+ with PAH uptake, but they also suggest that an Na^+ gradient alone may not be the only driving force for the intracellular accumulation of PAH. Kasher *et al.* [5] found that Na^+-dependent uptake in rat BLMV could be altered to uphill transport in the presence of an opposing gradient of unlabeled PAH, and they proposed an Na^+ gradient-stimulated anion exchange mechanism. Eveloff [11] recently demonstrated that in rabbit BLMV an outward OH^- gradient could also provide for concentrative uptake in combination with an inward Na^+ gradient.

With regard to PAH transport across the luminal

† To whom correspondence should be addressed.

membrane, it has been questioned for some time whether the downhill movement from cell to lumen occurs by unmediated or by mediated diffusion. The first evidence for mediated transport in membrane vesicles was demonstrated by Kinsella *et al.* [9], using brush border membrane vesicles (BBMV) from dog kidney. In the presence of an Na^+ gradient they found saturable and probenecid-sensitive PAH uptake. Na^+-stimulated transport was also described in BBMV from rabbit [8,12] and rat [4]. Others were unable to observe a stimulation with Na^+ [3,6,13] and it was shown that uptake is stimulated by an inside-positive diffusion potential induced by the Na^+ gradient, rather than by a direct interaction of Na^+ with a PAH transporter [4,12]. Recently, uphill PAH uptake into BBMV from dog [13,15] and rat [16] was demonstrated in the presence of an inwardly directed H^+ gradient. Kahn *et al.* [13,16] showed that stimulation by a pH gradient resulted from carrier-mediated PAH/OH^- exchange. This anion exchanger also accepts urate, and it can drive uphill transport of PAH and urate by opposing gradients of OH^-, Cl^- and HCO_3^-.

The purpose of the present study was to provide additional information on the effects of an Na^+ or H^+ gradient on PAH transport in isolated membrane vesicles. The characteristics and specificity of PAH uptake in BLMV and BBMV from dog kidney cortex were studied.

MATERIALS AND METHODS

Isolation of membrane vesicles. Brush border (BBMV) and basolateral membrane vesicles (BLMV) were isolated from the same cortical tissue preparation according to procedures described by Sheikh *et al.* [17] and Windus *et al.* [18] with some modifications. Kidneys from Beagle dogs that became available from other, mainly surgical, experiments were used as starting material. Immediately after excision, the kidneys were perfused with ice-cold saline to remove residual blood. All subsequent steps of the procedure were carried out at 4°. After the kidneys were decapsulated, slices of approximately 5–7 mm thick cortex were cut off, weighed, minced and suspended at 10% w/v in 100 mM mannitol, 5 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) buffered to pH 7.4 with Tris and 0.2 mM phenylmethylsulfonylfluoride. The mixture was homogenized with a loose-fitting dounce apparatus (30 strokes) and, subsequently, with a Polytron homogenizer on setting 7 for three 30-sec pulses. The cortex homogenate was centrifuged at 2500 *g* for 15 min in a Heraeus Minifuge GL, and the supernatant was treated with 10 mM $CaCl_2$ for 20 min. After a second centrifugation step of 2500 *g* for 15 min, the resulting supernatant contained the crude BBMV fraction and the pelleted material contained BLMV and membranes from other cell organelles. The crude BBMV fraction was spun at 24,000 *g* for 20 min in an IEC B-60 ultracentrifuge (A-169 rotor). The supernatant was discarded and the upper fluffy layer of the pellet was resuspended in 100 mM mannitol and 5 mM HEPES–Tris pH 7.4, homogenized with a tight-fitting dounce apparatus (30 strokes), and treated again with 10 mM $CaCl_2$ for 20 min. The pellet obtained after centrifugation at 2500 *g* for 15 min was discarded and the purified BBMV were collected at 30,000 *g* for 20 min. The BBMV pellet was washed with the appropriate uptake buffer, generally consisting of 100 mM KCl, 100 mM mannitol and 5 mM HEPES–Tris pH 7.4, to which 0.5 mM ethyleneglycol-bis-(β-aminoethyl ether)N,N'tetraacetic acid (EGTA) was added, centrifuged at 30,000 *g* for 20 min and finally resuspended in uptake buffer through a 23-gauge needle to obtain a final protein concentration of 8–12 mg/ml.

The pellet obtained after the first $CaCl_2$-precipitation step, containing the BLMV fraction, was resuspended in 250 mM sucrose and 5 mM HEPES-Tris pH 7.4, homogenized using a tight fitting dounce (30 strokes) and spun at 24,000 *g* for 20 min. The fluffy upper layer of the pellet was gently resuspended in the sucrose buffer to a volume of approximately 2 ml/g of initial cortex, homogenized with a tight fitting dounce (30 strokes) and brought to a concentration of 8% v/v Percoll. BLMV were separated from other cellular membranes on the self-orienting Percoll density gradient by centrifugation at 50,000 *g* for 30 min in an SB-283 rotor. A density gradient ranging from 1.020 to 1.050 g/ml as measured by a Bausch and Lomb refractometer was obtained and the purified BLMV were recovered from the second opaque band from the top of the gradient corresponding to a density of approximately 1.034 g/ml. The BLMV suspension was diluted in a ratio of 1 to 4 ml with the appropriate uptake buffer (generally 100 mM KCl, 100 mM mannitol and 5 mM HEPES–Tris pH 7.4) and the Percoll was removed by centrifugation at 96,000 *g* for 30 min. The fluffy layer on top of the glassy Percoll pellet was resuspended in uptake buffer by passing it through a 23-gauge needle. The BLMV were resuspended at a protein concentration of 8–12 mg/ml in uptake buffer.

The purity of the membrane preparations was assessed by assaying the specific activity of the following marker enzymes according to previously published procedures: alkaline phosphatase [19] and maltase [20] for brush border membranes, (Na^+–K^+)-ATPase [19] for basolateral membranes, succinate dehydrogenase [21] for mitochondria, acid phosphatase [22] for lysosomes and NADPH-dependent cytochrome-*c* reductase [23] for smooth endoplasmic reticulum. (Na^+–K^+)-ATPase was determined after detergent treatment with 0.4 mg/ml deoxycholate, followed by freezing-thawing, to disrupt membrane vesicles [18]. Protein was assayed with a commercial coomassie blue kit (BioRad, Müchen, F.R.G), with bovine plasma globulin as the standard. Compared to the initial homogenate, BBMV were enriched for maltase and alkaline phosphatase 12- to 14-fold, while the enrichment factors for the other marker enzymes were all <0.9. The results of the enzymatic analysis of BLMV showed an 8–10-fold enrichment in (Na^+–K^+)-ATPase, 0.8-fold in maltase, 2.5-fold in alkaline phosphatase, 1-2-fold in acid phosphatase and <0.8-fold in succinate dehydrogenase and NADPH-cytochrome-*c* reductase.

The membranes were rapidly frozen in liquid nitro-

gen and stored at −80° in small aliquots until used. In preliminary experiments, we found no difference in transport of glucose and PAH between frozen and freshly prepared membrane vesicles.

Transport studies. The uptake of D-[^{3}H]glucose, [^{3}H]glycine and [^{3}H]PAH in BBMV and BLMV was measured at 37° by a rapid filtration technique [24]. Measurement of solute uptake in membrane vesicles was initiated by the addition of 40 μl of solution to 10 μl of membrane suspension such that the initial content of the extravesicular medium was 100 mM NaCl, 20 mM KCl, 100 mM mannitol, 5 mM HEPES–Tris, pH 7.4 or 5 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES)–Tris pH 6.0; or 100 mM KCl, 100 mM mannitol, 5 mM HEPES–Tris pH 7.4 or 5 mM MES–Tris pH 6.0, and radiolabeled solute. The experimental conditions and the exact composition of the transport buffers are given in the legends. The initial solute concentrations were: glucose 25 μM, glycine 50 μM and PAH 50 μM or 100 μM except in concentration-dependence studies. The uptake of solute was terminated at appropriate time intervals by diluting the incubation mixture with 3 ml ice-cold stop buffer that had the same composition as the incubation medium but without the solute. This sample was immediately filtered under vacuum through a prewetted 0.45 μm cellulose nitrate filter (Schleicher and Schüll, Dassel, F.R.G.) and washed twice with 3 ml of ice-cold stop buffer. The radioactivity remaining on the filters was counted using standard liquid scintillation techniques after dissolution in 10 ml Aqualuma plus (Lumac, Schaesberg, The Netherlands). Corrections were made for the radioactivity bound to the filters in the absence of vesicles.

To ascertain whether PAH uptake by the vesicles represented transport across the membrane into the vesicles rather than binding to the membrane surface, PAH uptake at equilibrium was measured by increasing the extravesicular osmolarity with sucrose. Uptake of PAH was found to decrease linearly with the reciprocal medium osmolarity, and binding was estimated by extrapolating the uptake to infinite osmolarity, which corresponded to approximately 1–3% for BBMV and 10–20% for BLMV of the uptake under standard conditions (±345 mOsM).

Data analysis and presentation. Absolute uptake of solute is expressed as picomoles or nanomoles per milligram of protein. All experiments were performed on at least three different membrane preparations. Data are expressed as means ± SE. The kinetic constants of PAH uptake in BBMV and BLMV were determined from the initial linear uptake values (15 sec) at various substrate concentrations. Total PAH uptake could be described as uptake via a Michaelis–Menten process in parallel with passive diffusion. The carrier-mediated or specific component of uptake was defined as the difference between uptake in the absence and presence of 5 mM probenecid. Inhibition curves of probenecid were analyzed assuming competitive inhibition to a one binding site model. Curve fitting was done by least-squares nonlinear regression analysis using the computer program NONLIN [25]. Student's *t*-test was used to determine statistical significance. The figures presented in this study were drawn with the DISSPLA computer package [26].

Chemicals. D-[1-^{3}H]glucose (15 Ci/mmol) was obtained from New England Nuclear (Dreieich, F.R.G.) and [2-^{3}H]glycine (10 Ci/mmol) and *p*-amino[^{3}H]hippuric acid (254 mCi/mmol) were from Amersham (Bucks, U.K.). Percoll was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), iodopyracet from Dagra NV (Diemen, The Netherlands) and amiloride was a gift from Merck Sharp & Dohme (Haarlem, The Netherlands). All other chemicals were purchased from either Sigma (St. Louis, MO) or Merck (Darmstadt, F.R.G.) and were of the highest grade available.

RESULTS

Functionality of the membrane vesicles

The isolated membrane vesicles were functionally evaluated by measuring the Na^+ gradient-dependent glucose and glycine uptake. Figure 1 shows the time course of 25 μM glucose uptake into BBMV and BLMV in the presence or absence of an inwardly directed Na^+ gradient. In the presence of a Na^+ gradient an overshoot above the equilibrium value (90 min) of the uptake of glucose into BBMV was observed, while the replacement of NaCl by KCl resulted in the complete disappearance of the overshoot. Uptake at 15 sec in the presence of a Na^+ gradient was 9.3-fold enhanced over that observed without Na^+ (205 ± 19 vs 22 ± 3 pmol/mg protein; $P < 0.001$). BLMV showed no concentrative glucose uptake in presence of an Na^+ gradient and the initial rate of uptake was not different from uptake in an Na^+-free medium (8.9 ± 0.6 vs 8.3 ± 2.1 pmol/mg protein,15 sec; $P > 0.5$). Therefore, contamination of the BLMV preparation by BBMV must be functionally negligible.

The functional integrity of the vesicles was further evaluated through the Na^+ gradient-dependent transport of glycine. Figure 2 depicts the uptake of 50 μM glycine into BBMV and BLMV as a function of time in presence or absence of an inwardly directed Na^+ gradient. The imposition of an Na^+ gradient resulted in a substantial transient accumulation of glycine in BBMV and to a lesser extent in BLMV. The initial uptake of glycine measured at 15 sec, as compared with the values measured under Na^+-free conditions, was 9.8-fold stimulated in BBMV (161 ± 14 vs 16.5 ± 1.4 pmol/mg protein; $P < 0.001$) and 2.3-fold in BLMV (49 ± 4 vs 21 ± 3 pmol/mg protein; $P < 0.005$). These findings are in accordance with previously reported observations for glycine transport in dog kidney membrane vesicles [24]. Together with the glucose uptake data these results demonstrated the transport capability of our membrane vesicles.

Na^+ *and* H^+ *gradient-dependent PAH uptake*

The effect of imposed Na^+ or H^+ gradients on the uptake of 50 μM PAH into BBMV is illustrated in Fig. 3. In the right panel it is shown that when the extravesicular medium was at pH 6.0 and the intravesicular medium at pH 7.4 (6.0/7.4) the uptake of PAH was stimulated and a small transient accumu-

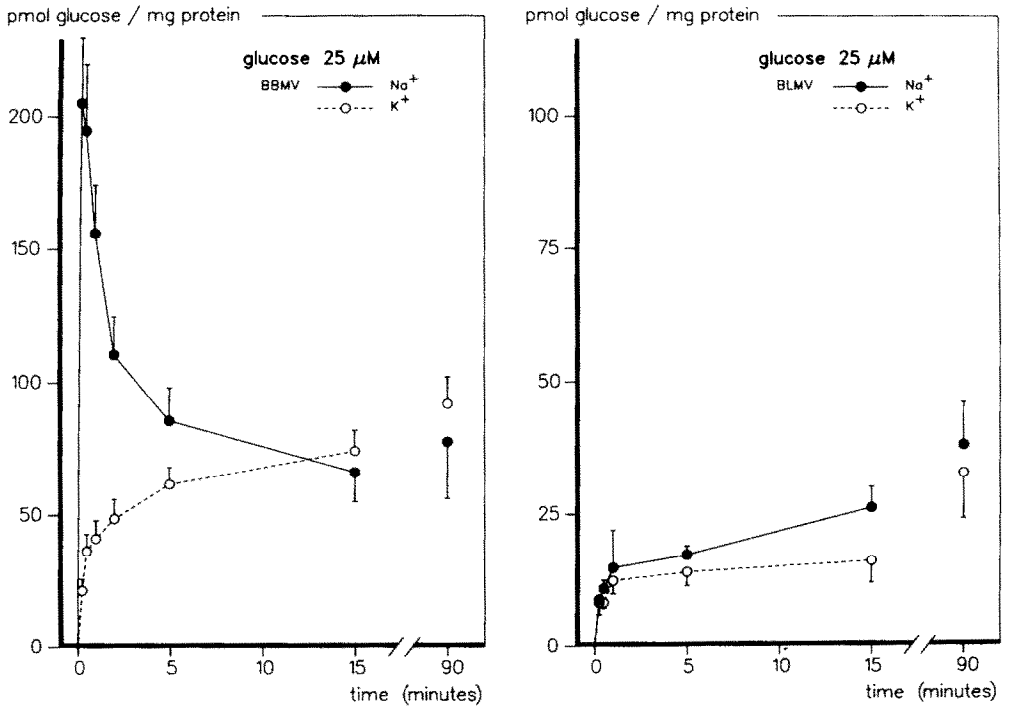

Fig. 1. Uptake of 25 μM glucose into BBMV (left panel) and BLMV (right panel) in the presence or absence of a Na^+ gradient. The vesicles were suspended in 100 mM KCl, 100 mM mannitol, 5 mM HEPES–Tris pH 7.4. The initial content of the extravesicular medium was 100 mM mannitol, 5 mM HEPES–Tris pH 7.4 and either 100 mM KCl or 100 mM NaCl and 20 mM KCl. Values are expressed as means ± SE for four experiments.

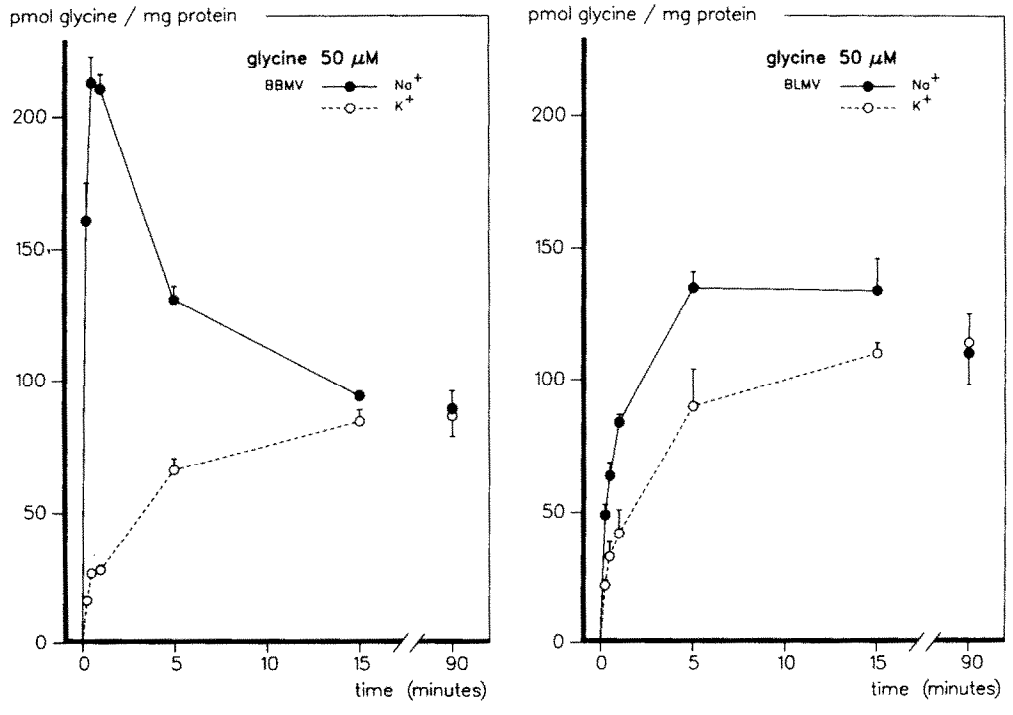

Fig. 2. Uptake of 50 μM glycine into BBMV (left panel) and BLMV (right panel) in the presence or absence of a Na^+ gradient. The experimental conditions were the same as in Fig. 1. Values are expressed as means ± SE of three experiments.

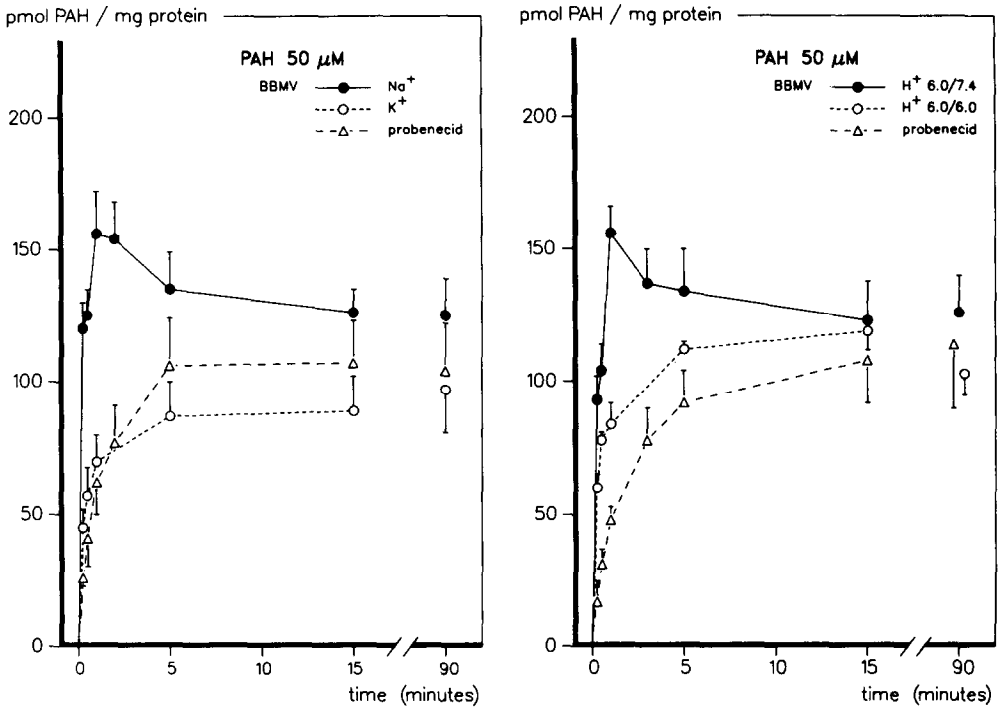

Fig. 3. Effect of a Na^+ gradient (left panel) or a H^+ gradient (right panel) on the uptake of 50 μM PAH into BBMV. Left panel: vesicles were suspended in 100 mM KCl, 100 mM mannitol, 5 mM HEPES–Tris pH 7.4. The initial content of the extravesicular medium was 100 mM mannitol, 5 mM HEPES–Tris pH 7.4 and either 100 mM KCl or 100 mM NaCl and 20 mM KCl with or without 5 mM probenecid. Values are expressed as means ± SE of five experiments. Right panel: vesicles were suspended in 100 mM KCl, 100 mM mannitol and either 5 mM HEPES–Tris pH 7.4 or 5 mM MES–Tris pH 6.0. The initial content of the extravesicular medium was 100 mM KCl, 100 mM mannitol, 5 mM MES–Tris pH 6.0. Uptake in presence of a H^+ gradient (H^+ 6.0/7.4) was measured with or without 5 mM probenecid. Values are expressed as means ± SE of four experiments.

lation over the equilibrium value was observed at 1 min. The uptake at 6.0/6.0 was slightly faster than at 7.4/7.4 (left panel, open circles); however, uptake rates were clearly decreased compared to the pH gradient situation. To distinguish mediated transport from nonspecific uptake by simple diffusion, H^+-dependent uptake was measured in the presence of 5 mM probenecid. This resulted in a marked reduction of the uptake rate, the initial (15 sec) uptake of PAH was inhibited 82% by probenecid (97 ± 7 vs 17 ± 4 pmol/mg protein; $P < 0.001$). Our results are consistent with previous investigations demonstrating that PAH uptake in BBMV is driven by an outwardly directed OH^- gradient via an anion-exchange transport mechanism [13, 14].

However, in contrast with these studies we also observed a stimulation of PAH uptake into BBMV by an inwardly directed Na^+ gradient as is shown in the left panel of Fig. 3. Imposition of a Na^+ gradient enhanced PAH uptake above that measured under KCl-equilibrated conditions. Similar to H^+-stimulated transport, maximum uptake was observed at 1 min slightly exceeding the equilibrium value. Probenecid also clearly interfered with Na^+-stimulated transport. The PAH uptake measured at 15 sec was inhibited from 103 ± 7 to 26 ± 3 pmol/mg protein (76%, $P < 0.001$).

It has been supposed that Na^+-stimulated PAH uptake into BBMV is mediated by a non-specific effect on the membrane potential, rather than by transport via an Na^+-PAH cotransport system [13]. To investigate this the effect of valinomycin on PAH uptake in the presence of an Na^+ gradient was measured (Table 1). Due to the outwardly directed

Table 1. Effect of membrane potential on Na^+ gradient-stimulated PAH uptake into BLMV and BBMV

		PAH uptake (pmol/mg protein)	
	Valinomycin	15 sec	90 min
BLMV	–	49 ± 4	71 ± 5
	+	41 ± 2	68 ± 6
BBMV	–	94 ± 5	117 ± 10
	+	70 ± 7*	110 ± 12

Uptake of 50 μM PAH was measured. The vesicles were suspended in 100 mM KCl, 100 mM mannitol, 5 mM HEPES–Tris pH 7.4. Valinomycin (10 μg/mg protein) was added in ethanol (2 μg/μl). An equal concentration of ethanol (1%) was added in the controls. The initial content of the extravesicular medium was 100 mM NaCl, 20 mM KCl, 100 mM mannitol, 5 mM HEPES–Tris pH 7.4. Values are expressed as means ± SE of three experiments. *$P < 0.05$ vs control

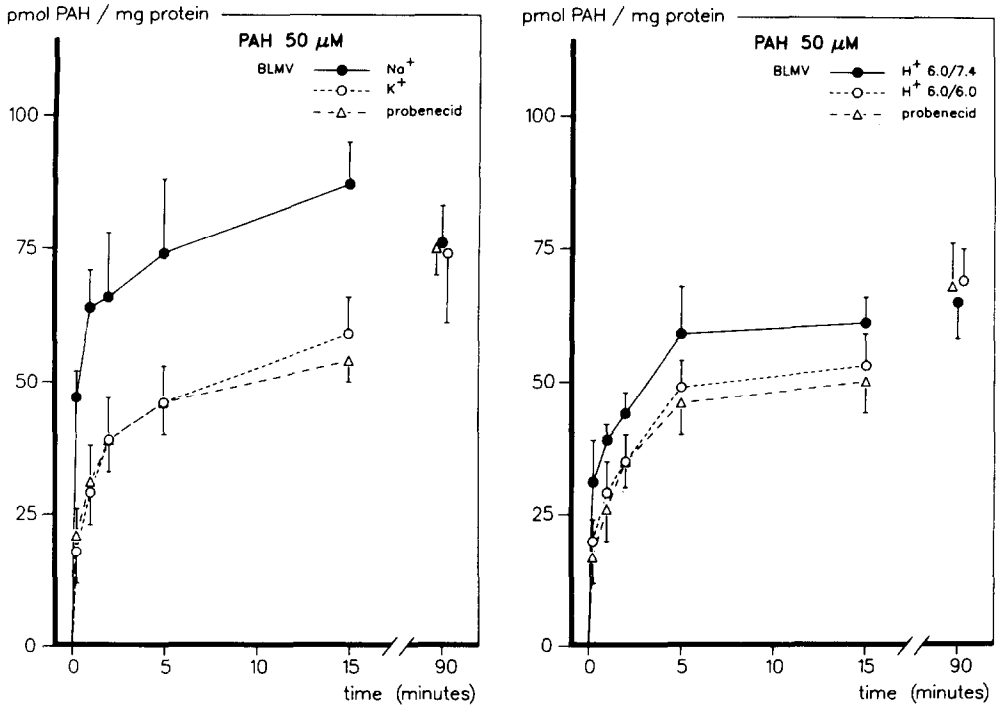

Fig. 4. Effect of a Na^+ gradient (left panel) or a H^+ gradient (right panel) on the uptake of 50 μM PAH into BLMV. The experimental conditions were the same as in Fig. 3. Values are expressed as means ± SE of four experiments.

K^+ gradient valinomycin renders the intravesicular space more electronegative [27]. This resulted in a significant reduction of PAH uptake into BBMV at 15 sec, confirming that at least an important part of Na^+ stimulated uptake into BBMV is mediated by an inside-positive membrane potential.

Figure 4 shows the effect of an Na^+ or H^+ gradient on the uptake of 50 μM PAH into BLMV. Uptake conditions were the same as described in Fig. 3. An inwardly directed H^+ gradient (6.0/7.4) slightly enhanced PAH uptake over that when the extra- and intravesicular pH were equal at 6.0 (Fig. 4, right

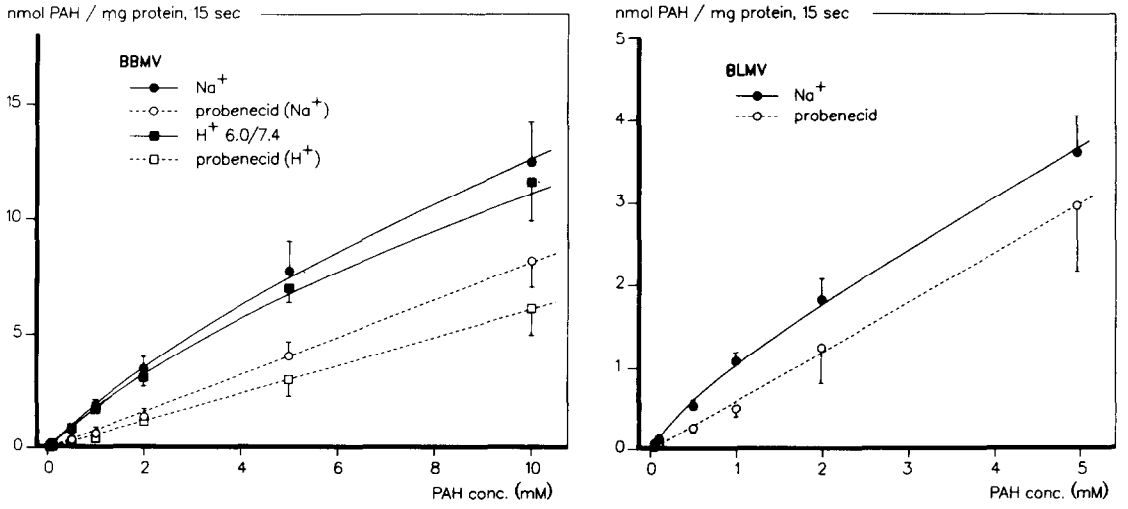

Fig. 5. Kinetics of Na^+ or H^+ gradient-dependent PAH uptake into BBMV (left panel) and Na^+ gradient-dependent uptake into BLMV (right panel). The vesicles were suspended in 100 mM mannitol, 5 mM HEPES–Tris pH 7.4. The initial content of the extravesicular medium was either 100 mM NaCl, 20 mM KCl, 100 mM Mannitol, 5 mM HEPES–Tris pH 7.4 or 100 mM KCl, 100 mM mannitol, 5 mM MES–Tris pH 6.0, in both cases with or without 5 mM probenecid. Uptake was measured at 15 sec in presence of increasing concentrations of PAH (0.5–10 mM). Values are expressed as means ± SE of three to five experiments.

Table 2. Kinetic parameters for Na^+ gradient-dependent PAH uptake into BLMV and Na^+ or H^+ gradient-dependent uptake into BBMV and the interaction with probenecid (prob)

	K_m (mM)	V_{max} (nmol/mg protein,15 sec)	$K_{i,prob}$ (mM)
BLMV (Na^+)	0.79 ± 0.16	0.80 ± 0.05	0.08 ± 0.01
BBMV (Na^+)	4.93 ± 0.57	6.71 ± 0.36	0.13 ± 0.01
BBMV (H^+)	5.72 ± 0.49	7.87 ± 0.33	0.16 ± 0.02

The experimental conditions were the same as those given in the legends to Figs 5 and 6. The results are means ± SE of three experiments.

panel) or at 7.4 (left panel, open circles), and no concentrative uptake was observed. Only a small inhibition was observed in the presence of probenecid (27 ± 5 vs 18 ± 3 pmol/mg protein,15 sec; $P > 0.10$), indicating that uptake occurred predominantly by simple diffusion.

In the left panel of Fig. 4, it is illustrated that the imposition of an Na^+ gradient resulted in a clear stimulation of PAH uptake into BLMV above that measured when no Na^+ was present. A small transient accumulation over the equilibrium value was observed after 15 min. Probenecid effectively inhibited the Na^+ gradient-stimulated uptake from 48 ± 4 to 20 ± 3 pmol/mg protein,15 sec (58%, $P < 0.005$). Valinomycin did not affect PAH uptake in the presence or absence of an Na^+ gradient (Table 1), suggesting that Na^+-dependent PAH uptake into BLMV is electroneutral and not influenced by the membrane potential.

Kinetics of PAH uptake and probenecid inhibition

The concept of carrier-mediated PAH transport was further supported by the saturability of Na^+ or H^+-dependent uptake into BBMV (Fig. 5, left panel) and Na^+-dependent transport into BLMV (Fig. 5, right panel) in the presence of increasing PAH concentrations. In both preparations concentration-dependent uptake could be described by a combination of carrier-mediated uptake following Michaelis–Menten kinetics and nonsaturable simple diffusion. The contribution of the non-specific component to total uptake was estimated from the amount of PAH uptake that could not be inhibited with 5 mM probenecid. The slopes of these linear curves were (nmol/mg protein,15 sec per mM) 0.811 ± 0.010 for BBMV (Na^+), 0.608 ± 0.007 for BBMV (H^+) and 0.595 ± 0.009 for BLMV (Na^+). The kinetic parameters characterizing the carrier-mediated, probenecid sensitive part of uptake (K_m and V_{max}) were derived from computer-based fit of the individual data and are given in Table 2.

The inhibitory effect of increasing probenecid concentrations on carrier-mediated PAH uptake into BLMV and BBMV is shown in Fig. 6. The inhibitory constants for probenecid (K_i) were calculated by curve fitting of individual data according to a one binding site model. Results are listed in Table 2. The results show that PAH transport in BBMV has a lower affinity, but a higher capacity than transport into BLMV. The kinetic parameters and probenecid inhibition constants for Na^+- and H^+-dependent transport are not significantly different from each other ($P > 0.1$), suggesting that both gradients stimulate PAH uptake via the same transport system.

Effect of various organic anions on PAH uptake

The substrate specificity of PAH transport into BLMV and BBMV was tested by *cis* inhibition experiments with various organic anions (Table 3). Each organic anion (2.5 or 5 mM) was added to the

Table 3. Effect of various organic anions on PAH uptake into BLMV and BBMV

		%PAH uptake		
Organic anion	mM	BLMV (Na^+)	BBMV (Na^+)	BBMV (H^+)
Probenecid	5.0	43 ± 3	29 ± 2	26 ± 9
Glycine	5.0	93 ± 5	101 ± 6	102 ± 7
Hippurate	5.0	69 ± 7	69 ± 9	77 ± 9
Phenolsulfonphthalein	5.0	64 ± 12	99 ± 9	44 ± 11
Iodopyracet	5.0	58 ± 9	92 ± 9	32 ± 8
Salicylate	5.0	54 ± 9	62 ± 3	54 ± 8
Acetylsalicylate	5.0	76 ± 11	78 ± 6	88 ± 12
Urate	2.5	87 ± 9	95 ± 6	70 ± 9
Hydrochlorothiazide	2.5	64 ± 12	69 ± 9	91 ± 13
1-Naphthyl-glucuronide	5.0	65 ± 12	81 ± 6	57 ± 12

The 15 sec uptake of 100 μM PAH was measured. The experimental conditions were similar to those given in the legend of Fig. 5, except for the presence of 2.5 or 5.0 mM organic anion. Results are expressed relative to the respective control uptakes as means ± SE of three experiments. Control uptakes, measured in presence of 5 mM gluconate, were (pmol/mg protein,15 sec) 111 ± 4 for BLMV (Na^+), 224 ± 10 for BBMV (Na^+) and 210 ± 18 for BBMV (H^+).

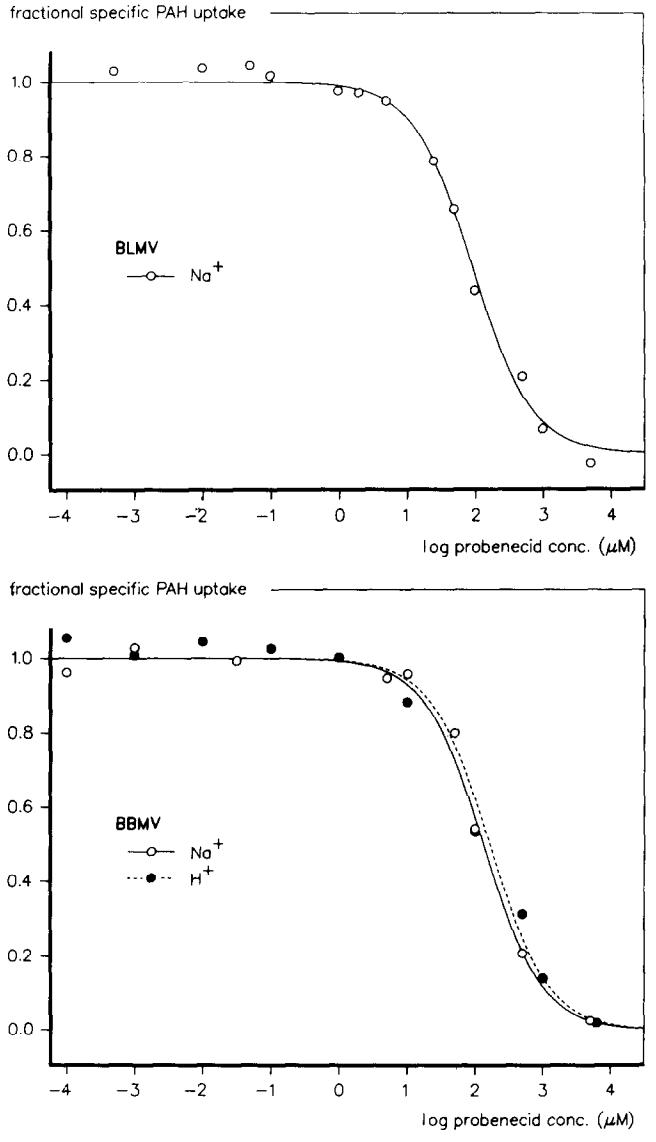

Fig. 6. Inhibition of specific Na^+ gradient-dependent PAH uptake into BLMV (upper panel) and Na^+ or H^+ gradient-dependent uptake into BBMV (lower panel) by probenecid. The vesicles were suspended in 100 mM KCl, 100 mM mannitol, 5 mM HEPES–Tris pH 7.4. The initial content of the extravesicular medium was 100 μM PAH and either 100 mM NaCl, 20 mM KCl, 100 mM mannitol, 5 mM HEPES–Tris pH 7.4 or 100 mM KCl, 100 mM mannitol, 5 mM MES–Tris pH 6.0, with varying probenecid concentrations (0.1 nM–5 mM). Uptake was measured at 15 sec and values are expressed as means of three experiments. SE were less than 10% for all values.

external medium and its effect on the 15 sec uptake of 100 μM PAH in the presence of an inward Na^+ (BLMV, BBMV) or H^+ gradient (BBMV) was measured. The strongest inhibition in both preparations was found with probenecid. Because PAH is a glycine conjugate the interaction with glycine was studied. Glycine had no effect on PAH uptake, and conversely we found no effect of concentrations up to 5 mM PAH on Na^+-dependent uptake of 50 μM glycine (data not shown), indicating that neither in BLMV nor in BBMV PAH and glycine share the same transport system. In BLMV iodopyracet and salicylate were good inhibitors, while urate only slightly affected PAH uptake. In BBMV some remarkable differences between the inhibitory effect on Na^+- and H^+-stimulated PAH uptake were observed. Phenolsulfonphthalein, iodopyracet and urate had no effect and 1-naphthyl-glucuronide had only a small effect on Na^+-stimulated uptake, whereas they clearly inhibited H^+-stimulated uptake. The effect of the other anions was virtually the same for both gradients with the exception of hydrochlorothiazide, which inhibited Na^+-stimulated uptake but did not interfere with uptake in presence of an H^+ gradient. The addition of acetate in concentrations up to 10 mM did not affect pH-dependent PAH uptake into BBMV. Since acetate diffuses rapidly into the vesicles, this indicates that

Table 4. Effect of an H^+ gradient on the time course of Na^+ gradient-dependent PAH uptake into BLMV and BBMV

Gradient	PAH uptake[a] (pmol/mg protein) 15 sec	1 min	90 min
BLMV			
Na^+ (control)[b]	45 ± 2	66 ± 7	76 ± 4
H^+[c]	29 ± 3*	44 ± 2*	65 ± 4
$Na^+ + H^+$[d]	68 ± 5*	97 ± 6*	82 ± 5
BBMV			
Na^+ (control)[b]	107 ± 5	156 ± 12	125 ± 8
H^+[c]	93 ± 10	156 ± 7	126 ± 14
Na^+[b] + amiloride (5 mM)	109 ± 6	149 ± 5	159 ± 14
$Na^+ + H^+$[d]	192 ± 6**	218 ± 9**	139 ± 9

[a]Uptake of 50 μM PAH was measured. The vesicles were suspended in 100 mM KCl, 100 mM mannitol, 5 mM HEPES–Tris pH 7.4. Values are expressed as means ± SE of three experiments.

The initial content of the extravesicular medium was:

[b]100 mM NaCl, 20 mM KCl, 100 mM mannitol, 5 mM HEPES–Tris pH 7.4,

[c]100 mM KCl, 100 mM mannitol, 5 mM MES–Tris pH 6.0,

[d]100 mM NaCl, 20 mM KCl, 100 mM mannitol, 5 mM MES–Tris pH 6.0.

*$P < 0.05$ and **$P < 0.01$ vs. respective controls.

the intravesicular buffering was sufficient to prevent the tested organic anions from influencing the transmembrane pH gradient [28]. Although the difference in pH may play a role by altering the degree of ionization of phenolsulfonphthalein ($pK_a = 7.9$), urate ($pK_a = 5.8$) and hydrochlorothiazide ($pK_a = 7.9$), this will hardly be of any influence on the differences in inhibitory effect observed for iodopyracet ($pK_a = 2.9$) and 1-naphthyl-glucuronide ($pK_a < 3$). Therefore, these results suggest that the pathways of Na^+ and H^+ gradient-stimulated PAH uptake into BBMV do not entirely overlap.

Effect of pH on Na^+-dependent PAH uptake

The effect of an H^+ gradient ($pH_{out} = 6.0$, $pH_{in} = 7.4$) on Na^+-dependent PAH uptake is shown in Table 4. With a pH gradient alone, uptake into BLMV was lower compared to Na^+ gradient-stimulated uptake, as was also noted in Fig. 4. Under pH gradient conditions Na^+-dependent PAH uptake into BLMV was significantly accelerated compared to the control where extra- and intravesicular pH were equal at 7.4. Valinomycin did not affect pH-stimulated Na^+-dependent uptake (data not shown). These results are similar to those observed by Eveloff [11] in the rabbit, who showed that PAH transport into BLMV occurs by an Na^+ gradient-stimulated OH^--exchange mechanism.

Because BBMV are known to contain an active Na^+–H^+ exchange system [29], Na^+-dependent uptake into BBMV was further evaluated by determining whether an inward directed Na^+ gradient indirectly stimulated PAH transport by generating an outward OH^-gradient. Uptake in the presence of the Na^+–H^+ exchange inhibitor amiloride (5 mM) was not different from the control (Table 4), indicating that Na^+–H^+ exchange does not play a role in Na^+-dependent PAH uptake. Simultaneous imposition of an H^+ and Na^+ gradient resulted in an almost 2-fold enhancement of the initial uptake rate compared to values measured when either an Na^+ or H^+ gradient was present alone. In the presence of valinomycin the 15 sec uptake was significantly decreased to 142 ± 11 pmol/mg protein ($P < 0.05$). Although this value was still higher than the control uptake the difference failed to reach the 5% level of significance. The results suggest that the PAH-OH^- anion-exchanger is stimulated by an inside-positive Na^+ potential. However, an alternative explanation would be that Na^+-dependent uptake and OH^--exchange represent two distinct transport pathways.

DISCUSSION

Basolateral membrane

Our findings demonstrate that PAH uptake into BLMV is mediated, functionally coupled to an Na^+ gradient across the membrane and insensitive to the membrane potential. These results are consistent with an electroneutral Na^+/PAH cotransport system in the basolateral membrane. Kinetic analysis demonstrated saturable probenecid-sensitive uptake in presence of an Na^+ gradient. The apparent K_m value determined for PAH was 0.79 mM. This value is in good agreement with previously reported values of 0.56 mM in the dog [9] and 0.54 mM in the rat [3], but considerably higher than the value of 0.054 mM also reported in rat [5]. The value for V_{max} of 0.80 nmol/mg protein,15 sec is somewhat high compared to previously reported values of (converted to nmol/mg protein,15 sec) 0.22 in the dog [9] and 0.11 [3] or 0.06 [5] in the rat. The K_i value for probenecid (0.08 mM) is again in good agreement with values of 0.14 mM [9] and 0.054 mM [3] reported for dog and rat BLMV, respectively.

In accordance with the wide variety of organic anions that are secreted *in vivo* by the kidney, vari-

ous compounds showed affinity for Na^+ gradient-stimulated PAH uptake in BLMV (Table 3). Of the compounds tested, only the endogenous anions glycine and urate hardly interfered with PAH transport. The lack of inhibition by urate supports the findings of Kahn *et al.* [30] in the rat that in contrast to the brush border membrane, the basolateral membrane seems to contain separate transport systems for PAH and urate.

The fact that a large Na^+ gradient is not able to achieve uphill transport in BLMV does not necessarily mean that an Na^+ gradient cannot drive the intracellular accumulation of PAH *in vivo*. In isolated membrane vesicles an overshoot is only achieved when V_{max} is relatively high compared to the rate of Na^+ gradient dissipation, whereas *in vivo* the transmembrane Na^+ gradient is always present. An alternative pathway for transport across the basolateral membrane might be an anion exchange mechanism similar to that described for PAH in the brush border membrane [13, 16]. However, in the presence of an outwardly directed OH^- gradient alone PAH uptake was only slightly stimulated, but when simultaneously an inward Na^+ gradient was imposed uptake was greatly stimulated. Our results provide supporting evidence for the Na^+ gradient-stimulated anion exchanger hypothesized by Kasher *et al.* [5], although we cannot fully exclude the possibility that uptake is the result of additive effects of two separate pathways. Very recently, Eveloff [11] reported Na^+-dependent PAH/OH^- exchange in BLMV from rabbit kidney. She concluded that anion exchange is directly coupled to Na^+, as it was found that PAH countertransport was also stimulated when Na^+ was equilibrated across the membrane.

What other anions than OH^- and PAH itself might be accepted by this anion exchanger remains to be examined. Löw *et al.* [31] proposed a multispecific anion exchanger in the basolateral membrane of the rat that could mediate exchange of sulfate for several inorganic and organic anions including PAH. This common exchanger could additionally be driven by an Na^+ gradient and a pH difference. However, capillary perfusion studies in the rat have indicated that the substrate specificities for PAH and sulfate transport into the tubular cell are clearly distinct [32].

Brush border membrane

The results of our transport studies in isolated BBMV indicate that PAH uptake can be stimulated by a transmembrane pH gradient (outward OH^- gradient) as well as by an inwardly directed Na^+ gradient. It is concluded that both gradients can drive PAH transport via a mediated pathway, since uptake in either case is concentration-dependent, probenecid-sensitive and susceptible to competition by other organic anions. Stimulation by an outwardly directed OH^- gradient is consistent with PAH/OH^- exchange mediated by the well recognized anion exchange mechanism in the brush border membrane that is also shared by urate [13–16]. Which transport system is involved in Na^+-stimulated uptake is less well understood, although it is generally accepted that Na^+ stimulation is not achieved via a cotransport mechanism but strictly by electrochemical effects [4, 12]. Hori *et al.* [4] proposed a gated channel through which PAH is transported in its anionic form driven by the transmembrane potential.

The question that arises from our experiments is whether an outward OH^- gradient and an Na^+-induced potential difference stimulate PAH uptake via the same carrier system or via separate carriers. The extra stimulation of PAH uptake by simultaneous imposition of both an Na^+ and a pH gradient provides little additional information. These results are as much consistent with additive effects of uptake via two distinct pathways as with stimulation of the PAH/OH^- exchanger by an Na^+-induced inside positive membrane potential. However, some evidence in favor of the latter mechanism comes from a study by Kahn *et al.* [16], who found a decrease in PAH/OH^- exchange when the intravesicular space was rendered more electronegative.

The fact that the kinetic parameters (K_m and V_{max}) and probenecid inhibition constant (K_i) for Na^+- and pH-dependent transport are virtually the same argues for a simple transport pathway. The parameters we found are, except for a higher V_{max} value, in good agreement with those observed by Kinsella *et al.* [9] for Na^+-stimulated PAH uptake in dog BBMV. They reported a K_m (apparent) of 3.87 mM, a V_{max} of 1.3 nmol/mg protein,15 sec and a K_i for probenecid of 0.30 mM. Surprisingly, this appears to be the only study reporting kinetic data on PAH transport in BBMV, and to our knowledge there are no data available allowing for a comparison of the kinetic parameters obtained in presence of a pH gradient.

On the other hand, the inhibition experiments with various organic anions show considerable, but not complete, overlap in the substrate specificities of Na^+- and pH-stimulated PAH transport. For some anions, e.g. phenolsulfonphthalein, urate and hydrochlorothiazide, the difference in inhibitory effect may be related with the degree of dissociation of the anion in the incubation medium. For these compounds, raising the pH from 6.0 to 7.4 greatly reduces the concentration of undissociated anion. However, the reduced inhibitory effects of phenolsulfonphthalein and urate at pH 7.4 are opposite to the enhanced inhibitory potency of hydrochlorothiazide.

At present no decisive answer can be given on the precise nature of the system(s) involved in Na^+- and pH-dependent PAH transport in BBMV, nevertheless our results are in favor of a simple transport pathway. In further studies it may be advisable to examine the dissipation of the imposed pH gradient or potential difference concomitantly with the transport of PAH. An important question also yet to be answered concerns the physiological relevance of these data. The anion exchanger present in the brush border membrane probably mediates active urate reabsorption in the proximal tubule, driven by anion gradients directed from cell to lumen. These gradients would also favor reabsorption of PAH, still PAH undergoes net secretion. It is presumed that the high intracellular PAH concentration as compared to the luminal concentration can overcome this reabsorptive tendency [28]. On the other hand, transport

mediated by a positive membrane potential, either via the anion exchanger, a gated channel or a leaky pathway, may very well reflect an important driving force that exists *in vivo*, as the luminal fluid has a more positive electrical potential than the intracellular compartment.

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