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p-Aminohippurate transport in basal-lateral membrane vesicles from rabbit renal cortex: stimulation by pH and sodium gradients

Jill Eveloff

Department of Physiology and Cardiovascular Resarch Institute, University of California, San Francisco and Nephrology Section, Veterans Administration Medical Center, San Francisco, CA 94121 (U.S.A.)

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_p_-Aminohippuric acid (PAH) uptake was studied in basal-lateral membrane vesicles prepared from rabbit renal cortex. An outwardly directed hydroxyl gradient ($pH_o = 6.0$, $pH_i = 7.6$) stimulated PAH uptake slightly over that when the internal and external pH values were equal at 7.6. A 100 mM sodium gluconate gradient directed into the basal-lateral membrane vesicles increased PAH uptake about 2-fold over that when _N_-methyl-D-glucamine or potassium gluconate gradients were present. When hydroxyl and sodium gradients were simultaneously imposed ($pH_o = 6.0$, $pH_i = 7.6$ and 100 mM sodium gluconate extravesicularly) PAH uptake was stimulated greater than with the pH or Na^+ gradient alone. In fact, an 'overshoot' was observed. Countertransport experiments showed that either intravesicular PAH or intravesicular PAH and Na^+ could stimulate ^{3}H-PAH uptake. Probenecid, an inhibitor of organic anion transport, inhibited both the hydroxyl-stimulated and Na^+ gradient-stimulated PAH uptake but the greatest inhibition by probenecid was seen when the hydroxyl and sodium gradients were both present. Thus, it is proposed that the driving force for PAH accumulation across the basal-lateral membrane of the proximal tubule is a transport system which moves Na^+ and PAH into the cell for an hydroxyl ion leaving the cell, i.e. a sodium-dependent anion-anion exchange system.

Introduction

The driving forces and mechanisms for the secretion of weak organic acids across the renal proximal tubule have been debated for 60 years. Since Marshall and Vickers demonstrated phenol red secretion in the nonfiltering dog kidney in 1923 [1], it has been recognized that organic acid secretion is active and carrier-mediated because tubule luminal concentrations of 600–1000-times that of the bath concentration can be achieved [2]. The carrier-mediated transport steps for secretion appear to be located at both the basal-lateral and brush-border membranes [3–11]. At the basal-lateral membrane, several transport mechanisms have been proposed. A direct coupling of the organic acid, *p*-aminohippuric acid (PAH) to the sodium gradient has been suggested by several investigators [12–17]. In metabolically poisoned rabbit kidney slices, a sodium gradient led to an accumulation of PAH greater than the final concentration found at equilibrium ('overshoot') suggesting a Na^+/PAH cotransport system in the basal-lateral membranes of the proximal tubule [15]. Sheikh and Moller [16] and Kahn et al. [17] have also observed stimulated PAH uptake in the presence of a sodium gradient directed into basal-lateral membrane vesicles from the rabbit and rat

Correspondence: J. Eveloff, Nephrology Section (111J), Veterans Administration Medical Center, 4150 Clement Street, San Francisco, CA 94121, U.S.A.

kidney. But several other laboratories have not observed this coupling and have suggested the stimulation by sodium was caused by its creating a positive electrical diffusion potential across the membrane which then increased the anion (PAH) uptake [3,10]. Recently, Kasher et al. found that PAH uptake into basal-lateral membranes was stimulated by a sodium gradient but that an 'overshoot' of PAH uptake only occurred in the presence of a sodium plus an opposing gradient of unlabeled PAH [10]. They suggested that the transport mechanism for PAH transport may be a sodium-dependent anion exchange system. Anion exchange systems have been previously described in the renal cortical basal-lateral membrane. Pritchard and Renfro [18] have described a sulfate/hydroxyl antiporter which was not sensitive to a sodium gradient in the rat kidney. However, Low et al. [19] observed a sulfate-hydroxyl anion exchange system in the rat basal-lateral membranes which was stimulated by a sodium gradient. Additionally, the anion exchange system had a broad specificity, accepting anions such as chloride, phosphate, mono-, di- and tricarboxylates as well as PAH.

The present studies were undertaken to reexamine the driving forces which effect PAH accumulation across the basal-lateral membrane of the proximal tubule, with particular attention to the role of pH and sodium gradients on PAH accumulation using basal-lateral membrane vesicles from the rabbit renal cortex.

Methods and Materials

Basal-lateral membrane preparation

Basal-lateral membranes from rabbit renal cortex were separated on sucrose density gradients essentially as described by Ives et al. [20]. The kidneys were removed from New Zealand White rabbits and perfused through the renal artery with cold ST buffer, containing (in mM): 250 sucrose, 10 Hepes-Tris (pH 7.6). The cortex was removed, placed in 80 ml ST buffer and homogenized 20 times with a glass/Teflon homogenizer, followed by three 30 s homogenizations with a Polytron (Brinkmann Instruments), speed 3, with one minute on ice between homogenizations. The homogenate was centrifuged at $1000 \times g$ for 15 min. The resulting supernatant was centrifuged at $35\,000 \times g$ for 30 min yielding a white fluffy pellet overlaying a brown pellet. The white fluffy pellet was gently resuspended in 80 ml of ST buffer and centrifuged at $48\,000 \times g$ for 30 min. The resulting white fluffy plasma membranes were suspended in 2.4 ml ST buffer and the membranes were run through a 25 gauge needle. The plasma membranes were mixed with 4.8 ml 70% sucrose buffered with 10 mM Hepes-Tris (pH 7.6) and placed in the bottom of two 40 ml ultracentrifuge tubes. Linear sucrose gradients were constructed on top of the plasma membranes, ranging in concentration from 35% to 48% sucrose buffered with 10 mM Hepes-Tris (pH 7.6) and spun 16 h at 28 000 rpm using a Beckman SW-28 rotor ($g_{av.} = 100\,000$) in an ultracentrifuge with the brake off. 2-ml fractions were collected using a wide-mouth pipet. The top six fractions were combined and diluted with 20 mM Hepes-Tris (pH 7.6) and the basal-lateral membranes were collected by centrifugation at $48\,000 \times g$ for 45 min. The basal-lateral membranes were suspended in a vesicle buffer containing generally (in mM): 200 sucrose, 1 magnesium gluconate, 20 Hepes-Tris (pH 7.6) and additions as noted in the figure legends.

The purity of the basal-lateral membranes was determined by assaying the activity of $(Na^+ + K^+)$-ATPase by the coupled assay of Schoner et al. [21]. Maltase activity was measured by following glucose release in the presence of maltase using the hexokinase reaction (Sigma kit No. 15-10) and the enzyme activity was used as a marker for luminal membranes. Succinate dehydrogenase activity was measured by the method of Ackrell et al. [22]. Protein was measured by the method of Lowry et al. [23]. The results of the enzymatic analysis indicated that the activity of the $(Na^+ + K^+)$-ATPase was enriched 16-fold compared to the enzyme activity of the homogenate, while maltase activity and succinate dehydrogenase activity were enriched 0.8- and 0.1-times, respectively. The basal-lateral membrane vesicles are derived from the several cell types found in the renal cortex and also contain small amounts of membranes from the endoplasmaic reticulum, Golgi, lysosomes and mitochondria [20]. It can not be ruled out that these membranes contribute to

the effect of the sodium and hydroxyl gradient on PAH transport.

Uptake of PAH by basal-lateral membrane vesicles

The uptake of ^{3}H-PAH was measured by the rapid filtration technique [6]. The ^{3}H-PAH was filtered prior to use through a 0.22 μm cellulose acetate filter to reduce nonspecific filter binding. The transport reaction was initiated by adding 20 μl of membranes to 130 μl of uptake buffer containing 15 μM ^{3}H-PAH (10 μCi), final concentration. The exact composition of the uptake and vesicle buffers and the experimental manipulations are described in the figure legends. All transport reactions were run at 25°C. The uptake of substrate was terminated at timed intervals by the removal of 20 μl of reaction medium and rapid dilution into 1 ml of cold stop solution, containing (in mM): 300 sucrose, 1 magnesium gluconate, 20 Hepes-Tris (pH 7.6). The diluted sample was rapidly filtered through a 0.65 μm cellulose nitrate filter (Sartorius No. SM11306) and washed once with 3 ml of cold stop solution. The cellulose nitrate filters bound less PAH than cellulose acetate filters. The filters were placed in scintillation fluid and counted by standard liquid scintillation techniques. All experiments were performed on at least four separate membrane preparations.

To estimate the percentage of PAH uptake which was bound to the basal-lateral membranes, uptake was studied by increasing the extravesicular osmolarity with raffinose. An estimate of binding was obtained by extrapolating the equilibrium uptake to infinite osmolarity, which corresponded to approximately 6% of the uptake measurement. Additionally, uptake at time zero was assessed by simultaneously mixing 1 ml of cold stop solution with 4 μl of basal-lateral membranes and 16 μl of the isotope-containing uptake buffer. The diluted sample was immediately filtered and washed with 3 ml of stop solution. The 'uptake' at time zero was the same as the background cpm.

Statistical analysis

The data are expressed as pmol/mg protein and given as means ± S.E. Significance between groups was tested by a paired Student's *t*-test. All experiments were performed on at least four separate membrane preparations.

Chemicals

N-Methyl-D-glucamine (NMG) gluconate was prepared by titrating *N*-methyl-D-glucamine with D-gluconic acid to pH 7.0. All salts were purchased from Sigma Chem. Co. (St. Louis, MO) and were of analytical grade. ^{3}H-PAH was obtained from New England Nuclear (Boston, MA).

Results

In the absence of sodium the uptake of 15 μM ^{3}H-PAH by renal cortical basal-lateral membrane vesicles as a function of time is shown in Fig. 1. An outwardly directed hydroxyl gradient (pH_o = 6.0, pH_i = 7.6) stimulated PAH uptake slightly over that when the inside and outside pH values were equal at 7.6, 16.8 ± 0.3 and 12.0 ± 0.4 pmol/mg protein per 10 s ($P < 0.001$), respectively. A pH gradient acceleration of PAH uptake has also been observed in renal brush-border membrane vesicles by Blomstedt and Aronson [4]. However, in the brush-border membranes, an overshoot of PAH transport was observed, which was 4-fold stimulated over that of the equilibrium value compared to that observed in the absence of a pH gradient.

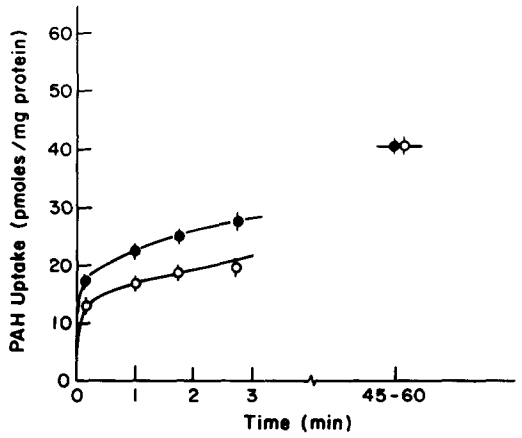

Fig. 1. The effect of a pH gradient on PAH uptake into basal-lateral membrane vesicles from rabbit renal cortex. The vesicle buffer contained (in mM): 200 sucrose, 50 potassium gluconate, 20 Hepes-Tris (pH 7.6), 1 magnesium gluconate, and 7 μM valinomycin (final concentration) was added to the membranes. The uptake buffer contained (in mM): 100 NMG gluconate, 50 potassium gluconate, 1 magnesium gluconate, 15 μM ^{3}H-PAH and 20 Hepes-Tris, pH 7.6 (○) or pH 6.0 (●). Values are the means ± S.E. of six experiments.

A sodium gradient directed into the basal-lateral membrane vesicles also increased PAH uptake above that when no sodium was present (Fig. 2). PAH uptake was 29.3 ± 0.9 pmol/mg protein per 10 s in the presence of sodium gluconate, 12.0 ± 0.6 pmol/mg protein per 10 s in the presence of NMG gluconate and 12.2 ± 0.5 pmol/mg protein per 10 s in the presence of potassium gluconate ($P < 0.001$). In these experiments, the intra- and extravesicular pH was equal at pH 7.6. The sodium gradient-stimulated PAH uptake was however small when compared to the effect of a sodium gradient on sulfate uptake in rat renal basal-lateral membranes [19].

When hydroxyl and sodium gradients were simultaneously imposed ($pH_o = 6.0$, $pH_i = 7.6$ and 100 mM sodium gluconate extravesicularly) PAH uptake was accelerated greater than the stimulation observed when either the sodium gradient or hydroxyl gradients were present alone (Fig. 3). In fact, a transient accumulation higher than the equilibrium value was observed, PAH uptake was 48.3 ± 0.2 pmol/mg protein per 10 s. The overshoot did not occur when the hydroxyl gradient was simultaneously imposed with other cation gradients such as potassium, NMG or lithium gluconate, PAH uptake was 15.4 ± 0.2, 16.4 ± 1.0 and 13.1 ± 1.2 pmol/mg protein per 10 s, respectively ($P < 0.001$). The results suggest that the sodium gradient may drive an anion exchange system for PAH and hydroxyl ions in the basal-lateral membrane.

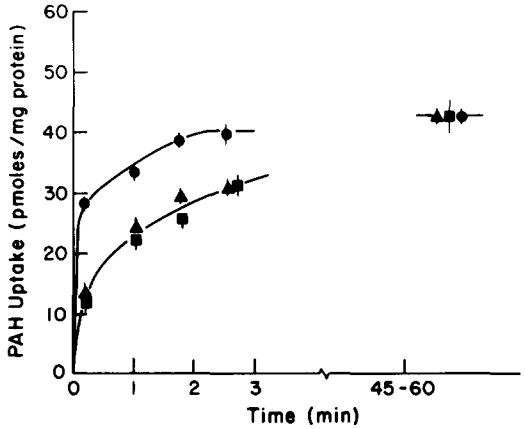

Fig. 2. Effect of a sodium gradient on PAH uptake into basal-lateral membrane vesicles from rabbit renal cortex. The vesicle buffer was the same as in Fig. 1. The uptake buffer contained (in mM): 100 sucrose, 20 Hepes-Tris (pH 7.6), 1 magnesium gluconate, 100 gluconate salt and 15 μM ^{3}H-PAH. The following gluconate salts were tested: sodium gluconate (●), NMG gluconate (■) and potassium gluconate (▲). Values are the means ± S.E. of four experiments.

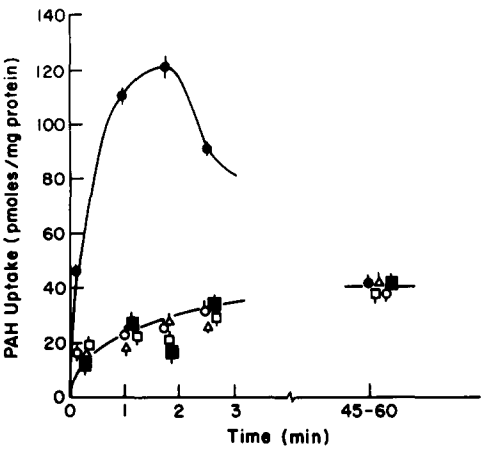

Fig. 3. The sodium gradient enhancement of hydroxyl-stimulated PAH uptake in basal-lateral membrane vesicles from rabbit renal cortex. The vesicle buffer was the same as in Fig. 1. The uptake buffer contained (in mM): 50 potassium gluconate, 20 Hepes-Tris (pH 6.0), 1 magnesium gluconate, 15 μM ^{3}H-PAH and one of the following salts: 100 sodium gluconate (●), 100 potassium gluconate (○), 100 NMG gluconate (▲), 100 lithium gluconate (□) or 200 mM sucrose (■). Values are the means ± S.E. of 4–6 experiments.

Countertransport experiments were conducted to give further evidence for a PAH carrier system in the basal-lateral membrane of the proximal tubule (Table I). The membranes were loaded with 200 μM PAH and 15 μM ^{3}H-PAH was added to the outside. The final extravesicular PAH concentration was 50 μM. Inside and outside pH values were equal at 7.6. Loading with PAH stimulated ^{3}H-PAH uptake 88% over that when the vesicles contained sucrose from 13.4 ± 1.2 to 25.6 ± 1.4 pmol/mg protein per 10 s, respectively ($P < 0.005$). If a 100 mM sodium gluconate gradient was added to the countertransport medium, ^{3}H-PAH uptake was greatly enhanced over the stimulation with the sodium gradient alone, from 28.4 ± 0.8 to 48.5 ± 2.3 pmol/mg protein per 10 s ($P < 0.005$) or 71% stimulation of uptake was observed. The sodium gradient stimulated countertransport, increasing PAH uptake from 25.6 ± 1.4 to 48.5 ± 2.3 pmol/mg protein per 10 s ($P <$

0.005), an increase of 90%. These results suggest that a transport system exists in the basal-lateral membrane which is stimulated by a sodium gradient. To test whether sodium interacted directly with the PAH transport system, the basal-lateral membranes were preequilibrated with 100 mM sodium gluconate. In Table I, PAH uptake was the same in the presence of a sodium gradient or in the presence of sodium on both sides of the membrane, 28.4 ± 0.8 and 27.6 ± 2.6 pmol/mg protein per 10 s, respectively ($P > 0.05$). However, if Na^+ was present on both sides of the membrane plus PAH was loaded into the vesicle, PAH uptake was 98.7 ± 5.8 pmol/mg protein per 10 s ($P < 0.001$). This data suggests that it is Na^+ per se which stimulates the PAH transport system and that sodium and PAH are cotransported.

The inhibition of PAH uptake by 2 mM probenecid is shown in Table II. When sodium or hydroxyl gradients are not present, probenecid inhibited PAH uptake only slightly, from 12.8 ± 1.3 to 11.3 ± 0.1 pmol/mg protein per 10 s ($P > 0.5$). When either a sodium gradient or hydroxyl gradient was present, the inhibition by probenecid was greater. The sodium gradient-stimulated PAH uptake was inhibited from 28.4 ± 0.8 to 15.4 ± 0.5 pmol/mg protein per 10 s ($P < 0.001$) or a 46% decrease. Similarly, the hydroxyl-stimulated PAH uptake was reduced from 17.3 ± 0.6 to 8.2 ± 1.2 pmol/mg protein per 10 s ($P < 0.005$). The largest probenecid inhibition of PAH uptake was observed when both the sodium and hydroxyl gradients were present, the PAH uptake was reduced from 46.2 ± 1.2 to 17.4 ± 0.3 pmol/mg protein per 10 s ($P < 0.001$).

The effect of membrane potential was tested on the sodium gradient-stimulated PAH uptake in order to test whether the PAH-anion exchange system is electroneutral (Table III). Under all conditions an inwardly directed potassium gradient was present. Valinomycin did not affect PAH uptake in the absence of a pH gradient across the vesicle or in the presence or absence of a sodium gradient. Valinomycin, in the presence of a potassium gradient, should create inside-positive diffusion potentials across the vesicle. Since previous experiments have shown that valinomycin did

TABLE I

THE EFFECT OF SODIUM ON PAH INFLUX AND COUNTERTRANSPORT INTO RENAL BASAL-LATERAL MEMBRANE VESICLES

The membrane vesicles were prepared in 100 mM sucrose, 1 mM magnesium gluconate, 50 mM potassium gluconate, 20 mM Hepes-Tris (pH 7.6), 7 μM valinomycin (final concentration) was added to the membranes. When indicated the vesicles were loaded with 200 μM unlabeled PAH or 100 mM sodium gluconate. The uptake buffer contained 100 mM sucrose, 1 mM magnesium gluconate, 50 mM potassium gluconate, 20 mM Hepes-Tris (pH 7.6), 15 μM ^{3}H-PAH, and when indicated 100 mM sodium gluconate or 200 mM sucrose for an osmotic control. Values are the means ± S.E.

External medium	Vesicular contents	PAH uptake (pmol/mg protein per 10 s)
^{3}H-PAH	sucrose	13.4 ± 1.2
^{3}H-PAH	PAH	25.6 ± 1.4
Na gluconate + ^{3}H-PAH	sucrose	28.4 ± 0.8
Na gluconate + ^{3}H-PAH	PAH	48.5 ± 2.3
Na gluconate + ^{3}H-PAH	Na gluconate	27.6 ± 2.6
Na gluconate + ^{3}H-PAH	Na gluconate + PAH	98.7 ± 5.8

TABLE II

PROBENECID INHIBITION OF PAH UPTAKE INTO RENAL BASAL-LATERAL PLASMA MEMBRANES

All values are the means ± S.E.

	PAH uptake (pmol/mg protein per 10 s) [a]	
	Control	2 mM probenecid
No sodium and no pH gradient [b]	12.8 ± 1.3	11.3 ± 0.1
Sodium gradient [c]	28.4 ± 0.8	15.4 ± 0.5
Hydroxyl gradient [d]	17.3 ± 0.6	8.2 ± 0.4
Sodium gradient and hydroxyl gradient [e]	46.2 ± 1.2	17.4 ± 0.3

[a] Basal-lateral membranes were prepared in 200 mM sucrose, 20 mM Hepes-Tris, 50 mM potassium gluconate and 1 mM magnesium gluconate.

[b] The uptake buffer contained 100 mM NMG gluconate, 50 mM potassium gluconate, 1 mM magnesium gluconate, 20 mM Hepes-Tris (pH 7.6) and 15 μM ^{3}H-PAH.

[c] The uptake buffer was similar to footnote (b) except 100 mM sodium gluconate was the salt.

[d] The uptake buffer was similar to footnote (b) except the pH was 6.0.

[e] The uptake buffer contained 100 mM sodium gluconate as the salt and the pH was 6.0.

TABLE III

EFFECT OF POTASSIUM DIFFUSION POTENTIALS ON SODIUM GRADIENT-STIMULATED PAH UPTAKE

The vesicle buffer contained 100 mM sucrose, 20 mM Hepes-Tris (pH 7.6), 1 mM magnesium gluconate plus or minus 7 μM valinomycin (final concentration, added to the membranes). The uptake buffer contained 50 mM potassium gluconate, 1 mM magnesium gluconate, 20 mM Hepes-Tris, pH 7.6 or 6.0, 15 μM ^{3}H-PAH and 100 mM sodium gluconate, if noted in the table. 200 mM sucrose was used as an osmotic control if sodium gluconate was absent. Values are the means ± S.E.

Extra-vesicular pH	Intra-vesicular pH	Sodium gradient	Valino-mycin	PAH uptake (pmol/mg protein 10 s per h)
7.6	7.6	−	−	13.4 ± 1.6
7.6	7.6	−	+	13.2 ± 0.8
7.6	7.6	+	−	27.8 ± 1.3
7.6	7.6	+	+	28.5 ± 0.8
6.0	7.6	+	−	50.4 ± 0.9
6.0	7.6	+	+	47.6 ± 1.2

stimulate ^{86}Rb flux into the vesicles [24], the absence of an effect of valinomycin on PAH uptake suggests the PAH-anion exchange system is electroneutral.

Discussion

The present study indicates that the driving force for PAH accumulation across the basal-lateral membrane of the renal cortex is a transport system which moves sodium and PAH into the cell for an hydroxyl ion leaving the cell, i.e. a sodium-dependent anion-anion exchange system. This transport system accounts for most of the experimental observations on the PAH transport system which have been made over the last 60 years. PAH uptake has been shown to be sodium-dependent in kidney cortical slices and inhibited by ouabain [12–15,25–27]. This has suggested that a sodium-coupled PAH cotransport system is in the basal-lateral membranes of the proximal tubule. Recently, Kahn et al. [17] and Kasher et al. [10] have shown stimulation of PAH uptake by a sodium gradient into rat and rabbit renal basal-lateral membrane vesicles.

Secondly, several weak organic acids are known to stimulate PAH secretion in low concentrations, such as acetate, lactate, pyruvate and alpha-ketoglutarate [28,29]. Since most of the original uptake studies were followed in renal slices after 35 to 120 min of incubation, it is possible that the effect is diffusion into the cell and stimulation of countertransport by the anion exchange system. Low et al. [19] have shown that sulfate uptake into rat basal-lateral membrane vesicles can be stimulated by intravesicular anions such as PAH, acetate, pyruvate and hydroxyl. They suggested the presence of a anion exchange system with a broad specificity in the basal-lateral membrane which was driven by the electrochemical gradient for Na^+ across the cell. The sodium gradient-stimulated PAH-anion exchange system described in this paper may be a part of the anion exchange system which was described by Low et al. [19].

The nature of the PAH cotransport system was recently investigated by two groups. Kasher et al. [10] demonstrated a sodium gradient-stimulated PAH-PAH countertransport system in basal-lateral membranes of the dog kidney. The results presented here confirm this observation and suggest that the countertransported ion with PAH may be the hydroxyl ion. They found, however, that if sodium was equilibrated across the vesicle that uptake was no greater that if the salt was not present suggesting that sodium may not be directly coupled to the anion exchanger. My data suggests a direct coupling of sodium with the PAH exchanger rather than an indirect effect since sodium, either as a sodium gradient or when sodium was equilibrated across the membrane, stimulated countertransport. Sheikh and Moller [16] have also demonstrated an electroneutral sodium-PAH cotransport in basal-lateral membranes from the rabbit kidney. I, however, was only able to demonstrate an overshoot when both a sodium and anion exchange gradient were imposed simultaneously. Thus, both this work and that of Kasher et al. proposed a sodium-dependent anion exchange mechanism for PAH transport. The stoichiometry of the PAH-anion exchange system was indirectly investigated by experiments which demonstrated the sodium gradient-stimulated PAH-anion exchange system was electroneutral. Electroneutrality of the Na^+/PAH cotransport system has also been demonstrated in the Newt kidney [13] and in basal-lateral membrane vesicles from the rabbit kidney [16]. In the

presence of sodium, electroneutrality could be maintained by transporting two PAH ions plus one sodium ion in exchange for one hydroxyl ion. Alternatively electroneutrality could be obtained by transporting one PAH ion, and one sodium ion in exchange for a hydroxyl ion and an unknown cation. The exact stoichiometry of the number of sodium ions to PAH ions remains to be determined.

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