J. Membrane Biol. 94, 279–284 (1986)

The Journal of
Membrane Biology

Ammonium Transport in Medullary Thick Ascending Limb of Rabbit Kidney: Involvement of the Na^+,K^+,Cl^--Cotransporter

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Summary. In order to investigate the question whether ammonium reabsorption in the thick ascending limb of Henle's loop (TALH) proceeds via the Na^+,K^+,Cl^--cotransporter, plasma membrane vesicles were prepared from TALH cells isolated from rabbit kidney outer medulla and the effect of NH_4^+ on their transport properties was investigated. It was found that, in the presence of a 78-mmol/liter NaCl gradient, 5 mmol/liter NH_4^+ inhibited bumetanide-sensitive rubidium flux by 86%; a similar decrease was observed for 5 mmol/liter, K^+. Inhibition of bumetanide-sensitive rubidium uptake by NH_4^+ was competitive and an apparent K_i of 1.9 mmol/liter was found. Bumetanide-sensitive sodium uptake measured in the presence of a 83 mmol/liter KCl gradient was not inhibited by 5 mmol/liter NH_4^+. A 100-mmol/liter NH_4Cl gradient was, however, capable of stimulating bumetanide-sensitive sodium uptake to the same extent as a KCl gradient. These data suggest that NH_4^+ is accepted by the K^+ site of the Na^+,K^+,Cl^--cotransport system and that the transporter can function in a $Na^+,NH_4^+,2Cl^-$ mode. Since the affinity of the transporter for NH_4^+ lies in the concentration range found in the TALH lumen in vivo, it is concluded that $Na^+,NH_4^+,2Cl^-$-cotransport can contribute to the NH_4^+ reabsorption in this tubular segment.

Key words ammonium · Na,K,Cl-cotransporter · potassium · thick ascending limb of Henle's loop

Introduction

Ammoniagenesis and ammonia secretion by the kidney play an important role in acid base homeostasis of the body (Pitts, 1973). Ammonia is secreted into the primary urine in the proximal tubule (Good & Burg, 1984) and is concentrated in the collecting duct for final excretion (Sajo et al., 1981). Until recently it was assumed that tubular transport of ammonia occurs predominantly, if not exclusively, by nonionic diffusion of NH_3 and can be accounted for by transepithelial pH differences. This view was challenged by Good, Knepper and Burg (1984) who demonstrated that in rat cortical thick ascending limb (TALH) absorption of the ammonium ion (NH_4^+) occurs against a transepithelial concentration gradient, excluding a role of nonionic diffusion in ammonia transport in this segment.

It was further observed that furosemide, an inhibitor of the Na^+,K^+,Cl^--cotransporter inhibited ammonia transport. Based on these observations the possibility was raised that the Na^+,K^+,Cl^--cotransporter in the thick ascending limb might be involved in the transepithelial ammonium transport. Since in the perfused tubule preparation furosemide inhibits not only the cotransporter but decreases simultaneously the transepithelial electrical potential difference, a distinction between paracellular transport of NH_4^+ (driven by the electrical potential difference) and transcellular transport (via the Na^+,K^+,Cl^--cotransporter) was not possible.

Such a distinction, however, can be achieved in isolated plasma membrane vesicles where changes in driving forces and inhibition of the cotransporter by furosemide can be dissociated. Using plasma membrane vesicles prepared from isolated cells of the thick ascending limb of rabbit kidney outer medulla, we therefore investigated whether NH_4^+ can serve as substrate for the Na^+,K^+,Cl^--cotransporter and, if so, which cation NH_4^+ could substitute for.

In the following it is demonstrated that NH_4^+ can replace potassium on the Na^+,K^+,Cl^--cotransporter. These results support the view that the TALH cells are capable of performing secondary active NH_4^+ transport, which is driven by the sodium and chloride gradient across the luminal membrane.

Materials and Methods

Methods

Preparation of Cells from the Medullary Thick Ascending Limb of Henle's Loop

Cells from the medullary thick ascending limb of Henle's loop were isolated from rabbit kidney outer medulla according to a method described previously (Eveloff, Haase & Kinne, 1980). In short, the outer red medulla of rabbit kidney is minced and incubated at 37°C for 1 hr with 0.2% collagenase/0.25% hyaluronidase (wt/vol) in Joklik's buffer gassed with 95% O_2/5% CO_2. Then single cells are released from the tubule segments by eight treatments with 0.25% trypsin (wt/vol) for 20 min at room temperature with oxygenation. The medullary cells are then separated on a continuous Ficoll gradient (2.7–30.1% wt/wt) at 1,400 $\times$ g_{max} for 45 min. The TALH cells are located in the lower part of the gradient as identified by their (Na^+,K^+)-ATPase activity. The cells were frozen and stored at −70°C in ST-buffer (200 mmol/liter sucrose, 10 mmol/liter triethanolamine, adjusted to pH 7.6 with H_2SO_4). Usually, two cell preparations derived from 12 kidneys with an average protein content of 24 mg were used for one membrane preparation.

Preparation of the Plasma Membrane Fraction for Transport Studies

Plasma membrane vesicles were prepared from isolated TALH cells by a series of differential centrifugation steps as described previously (König, Ricapito & Kinne, 1983). The membrane pellet obtained after the last centrifugation was suspended in vesicle buffer (100 mmol/liter sucrose, 20 mmol/liter triethanolamine, pH adjusted to 7.4 with H_2SO_4) with the aid of a 25-gauge needle mounted to a 1-ml syringe. The protein concentration of the final membrane suspension was 4–6 mg per ml, the protein yield was about 1.5 mg per experiment.

The plasma membranes were assayed for (Na^+,K^+)-ATPase (*see below*) and alkaline phosphatase as described previously (Bode, Pockrandt-Hemstedt, Baumann & Kinne, 1974). Protein was measured by the method of Lowry (Lowry, Rosebrough, Farr & Randall, 1951) after precipitation of the samples with 10% trichloroacetic acid (wt/vol).

Transport Studies

Solute uptake into the plasma membrane vesicles was determined by a rapid filtration technique (König et al., 1983). The incubation medium generally contained 100 mmol/liter sucrose, 20 mmol/liter triethanolamine (pH adjusted to 7.4 with HNO_3), 1 mmol/liter $Mg(NO_3)_2$, 0.5 mmol/liter Na salt or Rb salt and 99.5 mmol/liter of another salt. The composition of the intra- and extravesicular compartments, if different, is indicated in the figure legends. The uptake was initiated by adding 20 μl of membranes kept at 0°C to 120 μl of incubation medium kept at 25°C containing 12 μCi of ^{22}Na or ^{86}Rb. To determine uptake within 2 sec the reaction was initiated by adding 20 μl of membranes kept at 25°C to 25 μl of incubation medium kept at 25°C containing the radioisotopes. The transport reaction was terminated at timed intervals by rapid dilution of the reaction mixture into 1 ml of cold stop solution (in mmol/liter): 100 sucrose, 1 $Mg(NO_3)_2$, 20 triethanolamine, at pH 7.4, 150 KNO_3. The diluted sample was immediately filtered onto a cellulose nitrate filter (Millipore, HAWP, pore size 0.45 μm), and the filter was washed rapidly with 3 ml of cold stop solution. The filters were placed in scintillation fluid and counted by standard liquid scintillation techniques. Filter blanks were determined by adding 20 μl of vesicle buffer instead of membranes to the incubation medium. The filter blank amounted to about 5% of the uptake observed at equilibrium and was subtracted when calculating the amount of isotope taken up by the membranes.

For statistical analysis the Students' t test for paired data was employed. Bumetanide-sensitive solute uptake was calculated as the difference between the total uptake and the uptake in the presence of 1×10^{-3} M bumetanide.

Preparation of the Plasma Membrane Fraction for Studies on the (Na^+,K^+)-ATPase

Plasma membranes were isolated from rabbit kidney outer medulla as a light microsomal fraction by differential centrifugation as described previously (Kinne, Kinne-Saffran, Schölermann & Schütz, 1986). The average (Na,K)-ATPase activity in the membrane fraction was 15 μmol/hr · mg protein.

Determination of (Na^+,K^+)-ATPase

The activity of Mg^{2+}-ATPase and (Na^+,K^+)-ATPase (E.C. 3.6.1.3) was measured in freeze-thawed membranes in 75 mmol/liter Tris buffer (tris(hydroxymethyl) aminomethane), pH 7.6, using two different assays: (i) with 6 mmol/liter $MgSO_4$, 100 mmol/liter NaCl and 20 mmol/liter KCl and (ii) with $MgSO_4$, NaCl, KCl and 2 mmol/liter ouabain in the incubation media. 3 mmol/liter Tris-ATP was used as substrate. After 30 min of incubation at 37°C, the samples were heated for 2 min in boiling water, chilled, and centrifuged (2 min at 14,000 U/min, microsystem Eppendorf). For the determination of the affinity of the enzyme to K^+ or NH_4^+ different concentrations of either NH_4Cl or KCl were added to the incubation medium.

Materials

^{22}Na (carrier-free) was obtained from DuPont de Nemours, NEN Product Division (Dreieich, FRG), and ^{86}Rb (carrier-free) from Amersham (Braunschweig, FRG). Bumetanide (sodium-free) was a gift from Karl Thomae GmbH, FRG. For low speed centrifugation, a Sorvall RC5B and SS34 rotor were employed; for high speed centrifugation, a Sorvall OTD 65B and AH627 swing-out rotor were used.

All chemicals used were of highest purity commercially available.

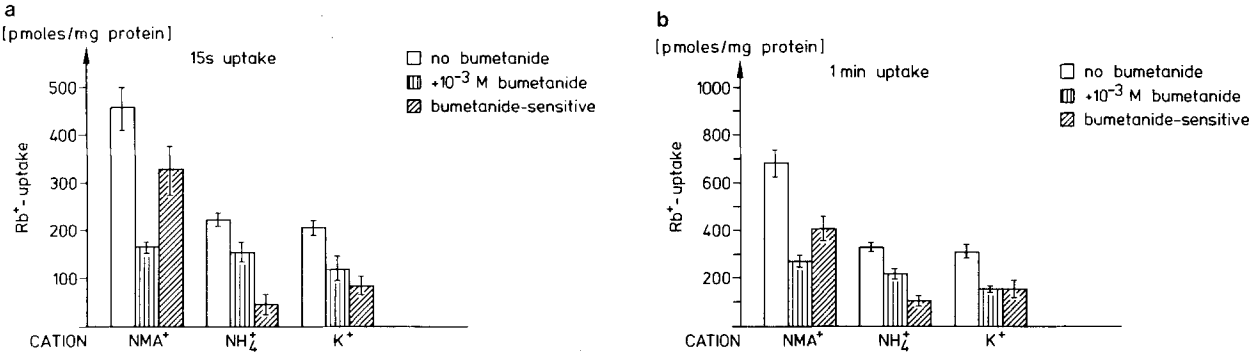

Fig. 1. Effect of ammonium and potassium on rubidium uptake by plasma membrane vesicles prepared from isolated TALH cells. □ total uptake, ▥ uptake in the presence of 1×10^{-3} M bumetanide, ▨ bumetanide-sensitive uptake. ^{86}Rb was used in a concentration of 0.5 mmol/liter, the concentration of ammonium and potassium was 5 mmol/liter. N-methylglucamine (NMA^+) was used as control. Uptake was determined in the presence of a 78-mmol/liter NaCl gradient as driving force. Mean values ± SEM derived from 4-6 determinations on three different plasma membrane fractions are depicted. (*a*) Uptake after 15 sec; (*b*) uptake after 1 min

Results

Effect of NH_4^+ on Rubidium Uptake by TALH Plasma Membrane Vesicles

In order to determine whether ammonium is capable of interacting with the potassium site of the Na^+,K^+,Cl^--cotransporter, first the effect of NH_4^+ on rubidium uptake was investigated in the presence of a constant driving force provided by a 78-mmol/liter NaCl gradient. At 0.5 mmol/liter radiolabeled rubidium, 5 mmol/liter NH_4^+ inhibited total rubidium uptake in the absence of bumetanide after 15 sec by 53% ($P < 0.01$) and after 1 min by 52% ($P < 0.01$) when compared to the uptake in the presence of 5 mmol/liter N-methylglucamine (*see* Fig. 1*a* and *b*). Rubidium uptake in the presence of bumetanide was only slightly affected by ammonium. Consequently bumetanide-sensitive uptake after 15 sec decreased by 86%, from 328 pmol/mg protein to 46 pmol/mg protein ($P < 0.05$), and after 1 min by 79% ($P < 0.002$). Figure 1*a* and *b* also show for comparison the effect of 5 mmol/liter K^+ on rubidium uptake. It can be seen that potassium has about the same inhibitory potency as ammonium on the bumetanide-sensitive rubidium uptake. There is also a small but significant ($P < 0.05$) reduction in the bumetanide-insensitive Rb^+ uptake by potassium.

In order to further characterize the interaction between the potassium site and NH_4^+, we investigated the effect of increasing concentrations of NH_4Cl on rubidium uptake at two different rubidium concentrations. When the results obtained are plotted according to Dixon (*see* Fig. 2), two straight lines are obtained which intercept above the abscissa. This result indicates that rubidium and ammonium compete for the same site (i.e., the K^+ site) of the transporter. When the results from three experiments were pooled, an average slope of 3.2 ± 0.6 and an average intercept with the ordinate of 9.7 ± 1.9 were obtained for 0.5 mmol/liter Rb and of

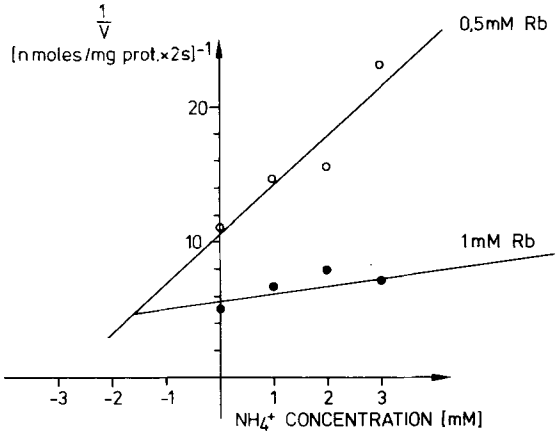

Fig. 2. Kinetics of the inhibition of bumetanide-sensitive rubidium uptake by NH_4^+. The effect of increasing concentrations of NH_4Cl on Rb^+ uptake was determined in the presence of 0.5 or 1 mmol/liter rubidium chloride and 100 mM NaCl as driving force. Uptake was measured for 2 sec to approach initial rates. Data are plotted according to Dixon. One representative experiment is shown

Table. Effect of NH_4Cl on bumetanide-sensitive sodium uptake in the presence of a 83 mmol/liter KCl gradient

	Incubation time			
	15″	1′	1′45″	2′30″
Control (triethanolamine)	279 ± 8.2	460 ± 17.1	582 ± 42.6	620 ± 48.7
NH_4Cl (4.1 mmol/liter)	363 ± 38.8	485 ± 39.6	542 ± 40.5	689 ± 86.25
LiCl (4.1 mmol/liter)	182 ± 22.9	253 ± 40.8	308 ± 47.5	317 ± 24.5

Data are given in pmol/mg protein and are expressed as mean values ± SEM derived from six determinations on three different plasma membrane preparations. Sodium concentration was 0.41 mmol/liter.

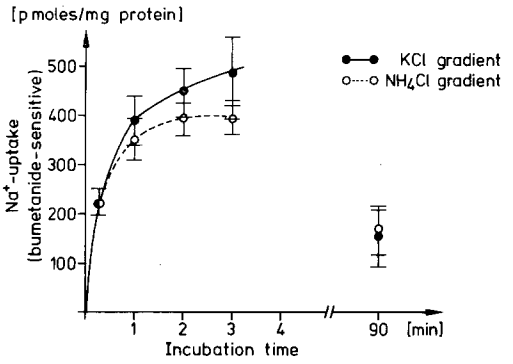

Fig. 3. Effect of an ammonium chloride gradient on bumetanide-sensitive sodium uptake by plasma membrane vesicles prepared from isolated TALH cells. The sodium concentration was 0.41 mmol/liter, the cation concentration 100 mmol/liter. Uptake in the presence of 100 mmol/liter KCl is given for comparison. Mean values ± SEM derived from 4-6 determinations on three different plasma membrane fractions are depicted

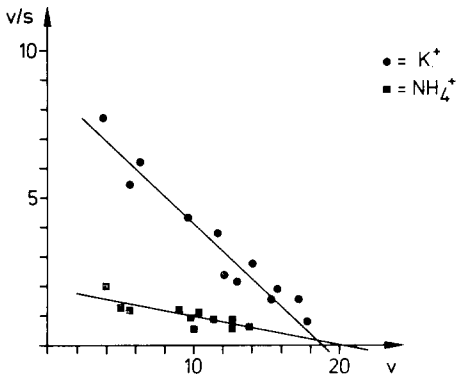

Fig. 4. Cation dependence of ouabain-sensitive ATPase activity in renal medullary plasma membranes. The assay contained (in mmol/liter) 100 NaCl, 6 $MgCl_2$, 3 ATP, 75 Tris buffer, at pH 7.6, and increasing concentrations of NH_4Cl or KCl. v = enzyme activity in μmol/hr · mg protein, s = concentration of the cation in mmol/liter. Data are derived from three experiments and are plotted according to Eadie Hofstee. Affinities were determined from the slope of the two straight lines by linear regression analysis

1.05 ± 0.2 and 5.7 ± 0.4 for 1 mmol/liter Rb. From these values an apparent affinity of the Na^+,K^+,Cl^--cotransporter for NH_4^+ of 1.9 mmol/liter can be calculated.

Effect of NH_4^+ on Sodium Uptake by TALH Plasma Membranes

The effect of NH_4^+ on sodium uptake by TALH plasma membrane vesicles was first investigated in the presence of a 83-mmol/liter KCl gradient as driving force. Under these conditions no effect of NH_4^+ on sodium uptake could be detected (*see* Table). Lithium, a common substitute for sodium in various sodium transport systems, however, inhibited sodium uptake significantly. This indicates that NH_4^+ interacts exclusively with the K^+ site of the Na^+,K^+,Cl^--cotransporter. In order to answer the question whether NH_4^+ can also be translocated by the cotransporter, we studied the effect of NH_4Cl gradient on bumetanide-sensitive sodium uptake by TALH plasma membrane vesicles. As shown in Fig. 3 bumetanide-sensitive sodium uptake in the presence of a NH_4Cl gradient is almost identical to the uptake in the presence of a KCl gradient. These data clearly indicate that NH_4^+ can replace K^+ at its binding site and is a substrate of the Na^+,K^+,Cl^--cotransporter.

Effect of NH_4^+ on (Na^+,K^+)-ATPase Activity in TALH Plasma Membrane

In order to investigate another potential transport system involved in transcellular transport of NH_4^+ (Kurtz & Balaban, 1986), we investigated the affinity of the outer medullary (Na^+,K^+)-ATPase to NH_4^+.

As shown in Fig. 4 in an Eadie Hofstee plot, (Na^+,K^+)-ATPase in outer medullary plasma membranes is stimulated halfmaximally by 2.1 mmol/

liter K^+. NH_4^+ has a much lower affinity for the enzyme, the apparent K_m is 10 mmol/liter, i.e., the affinity of the (Na^+,K^+)-ATPase for NH_4^+ under the experimental conditions employed is about five times lower than for K^+.

Discussion

Interaction of NH_4^+ with the Cation Binding Sites of the Na^+,K^+,Cl^--Cotransporter

The studies presented above provide evidence (i) that NH_4^+ inhibits the binding of rubidium to the potassium binding site of the Na^+,K^+,Cl^--cotransporter and (ii) that NH_4^+ can replace K^+ as a transportate during translocation. Interaction of NH_4^+ at the K^+-binding site is evident from the inhibition of Rb^+ flux by NH_4^+. This inhibition is fully competitive, suggesting that NH_4^+ inhibits Rb^+ transport by occupying the transport site rather than by interacting with another cation binding site, which may modify the properties of the transporter. The apparent affinity of the Na^+,K^+,Cl^--cotransporter for NH_4^+ in the presence of 100 mmol/liter NaCl was 1.9 mmol/liter. The high affinity is physiologically reasonable since NH_4^+ concentrations in the lumen of the TALH lie in a similar concentration range. The knowledge of the affinity of the cotransporter for NH_4^+ makes it also possible to estimate the apparent affinity of the transporter for potassium. From the results presented in the Dixon plot the concentration of NH_4^+ needed to inhibit Rb^+ flux by 50% can be calculated. It amounts to 3 mmol/liter at 0.5 mmol/liter Rb^+ and 5.8 mmol/liter at 1 mmol/liter Rb^+. From this result it follows that the affinity of the transporter for Rb^+ is sixfold higher than for NH_4^+, i.e., the $K_{m\mathrm{Rb}}$ is about 0.3 mmol/liter. Since in our experimental setup K^+ and Rb^+ behave very similar, it also can be assumed that $K_{m\mathrm{K}}$ is below 1 mmol/liter. Such a high K^+ affinity is consistent with the finding that the maximum effect of K^+ on transepithelial voltage in microperfused cortical TALH from rabbit kidney was observed at 4.8 mmol/liter, a concentration 10-fold higher than $K_{m\mathrm{K}}$ of the transport system (Greger, 1985).

It should also be noted that $K_{m\mathrm{K}}$ obtained in the current study is much lower than that found in our previous investigations (König et al., 1983). The reason for this discrepancy is probably that in the current studies a much higher sodium concentration was used (100 *vs.* 0.5 mmol/liter). The high sodium concentration saturates the sodium binding site ($K_{m\mathrm{Na}}$ = 1.3 mmol/liter at high KCl), and due to the high cooperativity between the sodium and the potassium binding sites (Saier & Boyden, 1984), the affinity of the potassium binding site of the transporter increases.

Evidence that NH_4^+, after *binding* to the cotransporter is *translocated* by the Na^+,K^+,Cl^--cotransport system comes from the observation that a NH_4^+ gradient is able to drive bumetanide-sensitive sodium transport. Since we are dealing with an experimental situation where a NH_4^+ gradient is established across the vesicle membrane, other possible effects of the NH_4^+ gradient on the transport system also have to be considered. First of all, the electrical potential difference across the membrane might have been altered when K^+ was replaced by NH_4^+. Such a change is very unlikely to affect the Na^+,K^+,Cl^--cotransporter because it has been shown that the cotransport has a stoichiometry of $1Na^+/1K^+/2Cl^-$ and is electroneutral (Greger, 1985; König et al., 1983). Furthermore, the linearity of the Dixon plot suggests a 1 : 1 interaction between NH_4^+ and the K^+ binding site. It therefore can be concluded that the translocation of Na^+ together with NH_4^+ and Cl^- follows the same stoichiometry of $1Na^+/1NH_4^+/2Cl^-$. Another possible effect of the NH_4^+ gradient may be a change in the intravesicular pH. However, neither the extent of pH change not its effect on the transporter can be presently predicted with any certainty. Despite these reservations, we would conclude from the similar time course of sodium uptake in the presence of the KCl and the NH_4Cl gradient that the two cations support bumetanide-sensitive sodium transport by an identical mechanism.

Transcellular Transport of Ammonia

If one attempts to transfer the results obtained with isolated plasma membranes to the intact cell, the following model for the active transcellular transport of ammonia in the medullary TALH emerges. At the luminal membrane ammonium enters the TALH cell via $Na^+,NH_4^+,2Cl^-$-cotransport. This cotransport could lead to an intracellular accumulation of ammonium until the driving forces of the sodium and chloride gradient that favor the cellular uptake of NH_4^+ are counterbalanced by the driving forces of the potassium and the ammonium gradient opposing the cellular uptake. The effective driving force for the accumulation would be equal to

$$-RT \ln \frac{[\mathrm{Na}]_i([\mathrm{K}^+]_i + 0.2[\mathrm{NH}_4^+]_i) \cdot [\mathrm{Cl}^-]_i^{2*}}{[\mathrm{Na}]_o([\mathrm{K}^+]_o + 0.2[\mathrm{NH}_4^+]_o) \cdot [\mathrm{Cl}^-]_o^2}.$$

This calculation takes into account that both potassium and ammonium compete for the K-site and that the affinity of the K-site for potassium is about fivefold higher than the affinity for ammonium. Due

* E. Heinz (*unpublished calculation*).

to the higher affinity of the transporter for K^+ theoretically very high intracellular concentrations of ammonium could be achieved. A rough calculation using $[Na^+]_i$ = 10 mM, $[Na^+]_o$ = 140 mM, $[K^+]_i$ = 155 mM, $[K^+]_o$ = 5 mM, $[Cl^-]_i$ = 30 mM, $[Cl^-]_o$ = 149 mM, and $[NH_4^+]_o$ = 4 mM would give a value of about 2,000 for the energetically possible distribution ratio of $[NH_4^+]_i/[NH_4^+]_o$. The actual intracellular ammonium concentration will, however, be determined by the fluxes of ammonium across the luminal and the contraluminal membrane, the degree of dissociation of ammonium in protons and ammonia, the relative permeabilities of the plasma membranes to ammonia, and the respective driving forces.

With regard to an efflux of ammonium across the luminal membrane our data suggest that recycling of ammonium via the barium-sensitive K^+ channel is probably small. The latter can be tentatively concluded from the fact that NH_4^+ inhibits bumetanide-insensitive rubidium uptake to a much lesser extent than potassium (*see* Fig. 1*b*).

With regard to the transport steps at the contraluminal membrane our studies provide no information about the mechanism(s) by which NH_4 can leave the cell. Depending on the actual concentration of NH_4^+ inside the cell, NH_4^+/Cl^- cotransport or NH_4^+/Na^+ exchange could be envisaged. Currently none of these processes has been studied in more detail.

Lastly, it has to be considered that ammonium can recycle across the peritubular membrane via the (Na^+,K^+)-ATPase. In order to get an estimate for the extent of the former process, we determined the apparent affinities of the medullary (Na^+,K^+)-ATPase for K^+ and NH_4^+. Our results show that at least under the conditions of the assay, the affinity of the enzyme for NH_4^+ is fivefold lower than the affinity for K^+. If the same is true for the situation in vivo even at high concentrations of NH_4^+ and K^+ in the medullary interstitium (Johnston, Battilana, Lacy & Jamison, 1977; Stern, Backman & Hayslett, 1985)—contrary to the predictions of Kurtz and Balaban (1986)—there would be a preference of the enzyme (and probably also of the transport) for K^+.

We express our appreciation to Prof. Dr. E. Heinz for valuable discussions, Ms. D. Mägdefessel for excellent secretarial assistance, and Mr. F. Dräger for his art work.

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Received 8 July 1986