Luminal Secretion of Ammonia in the Mouse Proximal Tubule Perfused in Vitro

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Abstract

A major portion of the total ammonia ($tNH_3 = NH_3 + NH_4^+$) produced by the isolated perfused mouse proximal tubule is secreted into the luminal fluid. To assess the role of Na^+-H^+ exchange in net tNH_3 secretion, rates of net tNH_3 secretion and tNH_3 production were measured in proximal tubule segments perfused with control pH 7.4 Krebs-Ringer bicarbonate (KRB) buffer or with modified KRB buffers containing 10 mM sodium and 0.1 mM amiloride. Net tNH_3 secretion was inhibited by 90% in proximal tubule segments perfused with the pH 7.4 modified KRB buffer while tNH_3 production remained unaffected. The inhibition of net tNH_3 secretion by perfusion with the modified KRB buffer was only partially reversed by acidifying the modified KRB luminal perfusate from 7.4 to as low as 6.2. These data indicate that the Na^+-H^+ exchanger facilitates a major portion of net tNH_3 secretion by the proximal tubule and that luminal acidification may play only a partial role in the mechanism by which the Na^+-H^+ exchanger mediates net tNH_3 secretion.

Introduction

Secretion of ammonia from within the proximal tubule cell into the luminal fluid is the initial step in ammonia handling by the nephron (1–4). Furthermore, the major fraction of the ammonia excreted by the nephron into the final urine is already present in the luminal fluid of the late proximal tubule. The factors regulating cell-to-lumen ammonia entry in the proximal tubule have not been fully explored because of the limitations inherent in techniques previously used. The standard free-flow micropuncture technique has been used to assess luminal ammonia entry in the past, but because total ammonia production rates cannot be measured using the micropuncture technique it is difficult to ascertain whether altered rates of luminal ammonia entry result from altered rates of production, from altered rates of transport, or from both. The in vitro microperfusion technique originally developed by Burg and colleagues (5) has been applied to examine transepithelial fluxes in various segments of the nephron including the proximal tubule (6, 7). Although this technique has provided important information about *transepithelial* ammonia flux, it does not directly examine the factors which regulate cell-to-lumen ammonia entry.

Recent studies from this laboratory have demonstrated that total ammonia ($NH_3 + NH_4^+$) production and net luminal total ammonia secretory rates may be determined in isolated perfused mouse proximal tubule segments (8, 9). The total ammonia production rate represents the rate at which total ammonia leaves the perfused proximal tubule segment via the peritubular and luminal aspects of the tubule. The luminal total ammonia output rate reflects the difference between the cell-to-lumen entry rate and the rate at which total ammonia leaves the luminal fluid via cell reentry or the paracellular pathway. Thus, the luminal total ammonia output rate represents the net rate of total ammonia secretion. In our previous studies, we demonstrated that most of the total ammonia produced by a perfused proximal tubule segment is secreted into the luminal fluid and enters the bath medium via the luminal fluid leaving the distal end of the perfused segment (8, 9). In the present study, we examined the roles of the Na^+-H^+ exchanger and intraluminal pH on net luminal total ammonia secretion and total ammonia production rates.

Methods

Animals. Male Swiss Webster mice (Hilltop Laboratories, Scottdale, PA) weighing 25 to 35 g had free access to Purina Rodent Chow (Ralston-Purina Co., St. Louis, MO) and water.

Isolation and perfusion of the individual proximal tubule segments. This method has been described in detail (8). Briefly, a mouse was killed by cervical dislocation and a kidney rapidly removed and placed in iced Krebs-Ringer bicarbonate (KRB) buffer. Proximal tubule segments consisting of the late convoluted and early straight portions from the outer cortex were dissected with needle-tipped forceps under microscopic guidance. Tubule segment lengths were measured with a calibrated eye-piece micrometer and ranged from 0.6 to 1.2 mm. The tubules were cannulated and perfused with KRB buffer. The perfusate buffer was pregassed at 37°C with 95% O_2: 5% CO_2 gas mixture and collected in gas-tight syringes that were mounted in a syringe pump (Sage Instruments, Cambridge, MA). The perfusates were delivered into the perfusion pipette without direct exposure to room air. The initial perfusate pH values reported below represent pH values of fluid leaving the perfusion pipette. The tubules were incubated at 37°c in ~ 150 μl of KRB buffer containing 0.5 mM L-glutamine and 1.0 mM sodium acetate pregassed with 95% O_2: 5% CO_2 gas to ensure adequate stirring. At the end of the incubation period an aliquot of the bath medium was taken for measurement of total ammonia. The final bath volume was determined from the dilution of known amounts of trypan blue dye added to the perfusion chamber.

In some experiments, a second pipette was used to collect the fluid leaving the distal end of the perfused segment. The total ammonia content in timed luminal fluid collections was used to determine net rates of total ammonia secretion.

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Total ammonia assay. Total ammonia was measured using the method developed in this laboratory (8). This method was based upon a series of enzymatic reactions that were ultimately coupled with a bioluminescence reaction so that light emission was directly proportional to the amount of total ammonia in the sample. Total ammonia in the sample quantitatively reacted with excess NADH and α-ketoglutarate in the presence of glutamic dehydrogenase to form glutamate and oxidized NAD (NAD^+). The excess NADH was destroyed by acid hydrolysis and the remaining NAD^+ was measured using a series of coupled enzymatic reactions involving glucose-6 phosphate dehydrogenase, NAD:FMN oxido-reductase, and bacterial luciferase. The light emission of the processed sample was measured on a photometer (model 20; Turner Designs, Mountain View, CA). The relationship between plateau light emission rate and total ammonia concentration was linear and the coefficient of variation in the range of total ammonia concentrations observed in our samples was 0.05 to 0.1.

Determination of luminal fluid pH. Luminal fluid pH was measured using a glass pipette (10 μm tip diam) in which a liquid ion exchanger specific for hydrogen ions (World Precision Instruments, New Haven, CT) was added to the presilanized tip and back-filled with sodium phosphate buffer pH 7.0 (10). The pipette was connected via Ag/AgCl wire to a high input impedance electrometer (FD 223; World Precision Instruments). The distal most portion of the electrode tip was desilanized with 10 M KOH so that a very small reservoir of water could be maintained at the tip thus making the electrode resistant to poisoning by mineral oil (11). The luminal fluid specimens were collected under pre-gassed mineral oil with a collection pipette. The liquid ion exchanger and KCl reference pipettes were directly introduced into the mineral oil and collected fluid, and advanced to the distal most portion of the collection pipette so that the tip of the liquid ion exchanger pipette was positioned at the luminal opening of the distal end of the perfused segment. The pH was determined from the difference between the voltages observed with the ion-specific and reference electrodes. Standard pH solutions measured with the two electrode system revealed a sensitivity of 56 mV/pH.

Solutions. The KRB buffer contained NaCl (125 mM), $NaHCO_3$ (25 mM), KCl (5 mM), $MgCl_2$ (1.0 mM), NaH_2PO_4 (1.0 mM), and $CaCl_2$ (1.0 mM). As described below, in some experiments, the sodium concentration of the perfusate buffer was reduced to 10 mM by isoosmotically replacing sodium chloride with mannitol and partially replacing sodium bicarbonate with choline bicarbonate (16 mM choline bicarbonate and 9 mM sodium bicarbonate). The pH of the perfusate was lowered in certain experiments by reducing the bicarbonate concentration and raising the chloride concentration by an equivalent degree.

Calculations. Total ammonia production rates (picomoles per minute per millimeter) were calculated from the bath total ammonia concentration, bath volume, incubation time, and tubule segment length, as described before (8). Luminal total ammonia output rates were calculated from the total ammonia content of timed luminal fluid samples (8). Since the luminal total ammonia output rate was the difference between the rate of total ammonia addition to the luminal fluid and the rate of total ammonia removal from the luminal fluid, the luminal total ammonia output rate represented the net total ammonia secretory rate. Flow rates were determined by the rate that known amounts of [^{3}H]methoxyinulin left the distal end of the perfused segment.

Data are presented as mean±SE. Comparisons between two groups of data were made using Student's t test and comparisons among more than two groups of data were made using a single analysis of variance with multiple comparisons among groups of varying size (Sheffé's method) (12).

Results

Under the experimental conditions, total ammonia production and net total ammonia secretion remained stable for up to 60 min of incubation at 37°C. All measurements were obtained within 60 min after dissection. All tubules excluded trypan blue. Flow rates in the groups of tubules ranged from 20.3 to 22.4 nl/min and there were no statistically significant differences in mean flow rates among the groups. The lengths of individual tubule segments ranged from 0.6 to 1.2 mm with no statistically significant differences in the mean segment length among the groups studied. The mean length of tubules in this study was 0.86 mm.

Total ammonia production and net total ammonia secretion rates were measured in tubules bathed in pH 7.4 KRB buffer containing 0.5 mM L-glutamine and 1.0 mM sodium acetate and perfused with the control pH 7.4 KRB buffer or with a modified KRB buffer in which 0.1 mM amiloride was added and the sodium concentration was reduced to 10 mM by isoosmotically replacing sodium chloride with mannitol and partially replacing sodium bicarbonate with choline bicarbonate. Perfusion with the modified KRB buffer containing 10 mM sodium and 0.1 mM amiloride inhibited the rate of fluid reabsorption by 5 proximal tubule segments (0.1±0.1 nl/min per mm with the modified KRB perfusate versus 0.8±0.2 nl/min per mm with control KRB perfusate) at flow rates of 21 nl/min. Perfusion with the modified KRB buffer also inhibited measurable luminal fluid acidification. The collected fluid pH was 7.27±0.01 in five tubules perfused with the control KRB buffer (initial pH 7.43±0.01) and was 7.44±0.01 in five tubules perfused with the modified KRB buffer (initial pH = 7.43±0.01) at flow rates of 21 nl/min. Proximal tubule segments perfused with the control buffer at a flow rate of 20.9±0.4 nl/min produced total ammonia at a rate of 21.0±0.5 pmol/min per mm, which was not significantly different from the rate observed in segments perfused with the modified KRB buffer at a flow rate of 21.0±0.5 nl/min, 21.0±0.4 pmol/min per mm (Fig. 1). The rate of net total ammonia secretion was 11.3±0.3 pmol/min per mm in tubules perfused with the control KRB perfusate, while it was only 1.0±0.2 pmol/min per mm in tubules perfused with the modified KRB buffer. Thus perfusion with the modified KRB buffer containing 10 mM sodium and 0.1 mM amiloride markedly diminished net total ammonia secretion without significantly affecting total ammonia production (Fig. 1).

To assess the importance of luminal acidity on total ammonia production and net secretion of total ammonia, proximal tubule segments were perfused with the 10 mM sodium buffer containing 0.1 mM amiloride in which the pH was lowered by reducing the bicarbonate concentration while maintaining a constant sodium concentration of 10 mM. The pH of the collected luminal fluid of perfused proximal tubule segments was measured when the segments were perfused with pH 7.4 KRB control perfusate (group I) or with the modified KRB buffers containing 10 mM sodium, 0.1 mM amiloride and either 25 mM (group II), 5 mM (group III) or 1.2 mM (group IV) bicarbonate (Table I). Perfusion flow rates did not differ among the groups averaging 21.2±0.5 nl/min. The pH of collected fluid samples of proximal segments perfused with the modified KRB buffer containing 1.2 mM bicarbonate was significantly lower than the tubules perfused with control KRB buffer ($P < 0.01$) or with the modified KRB buffer containing 25 mM bicarbonate ($P < 0.01$). The pH of luminal fluid samples of segments perfused with the modified KRB buffer containing 5 mM bicarbonate was significantly lower than the pH

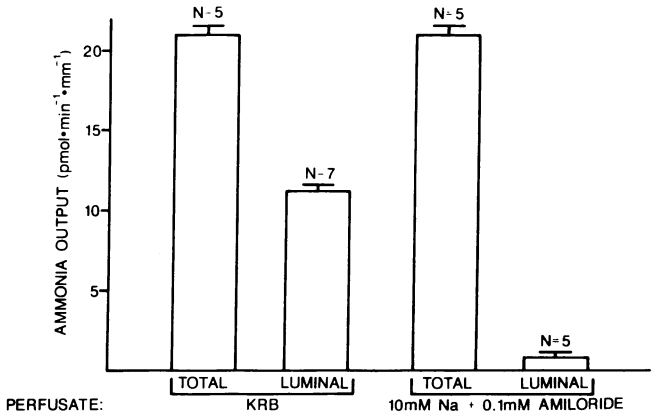

Figure 1. Luminal total ammonia output and total ammonia production rates by mouse proximal tubules bathed in pH 7.4 KRB buffer and perfused with either control pH 7.4 KRB buffer or with pH 7.4 modified KRB buffer containing 10 mM sodium and 0.1 mM amiloride.

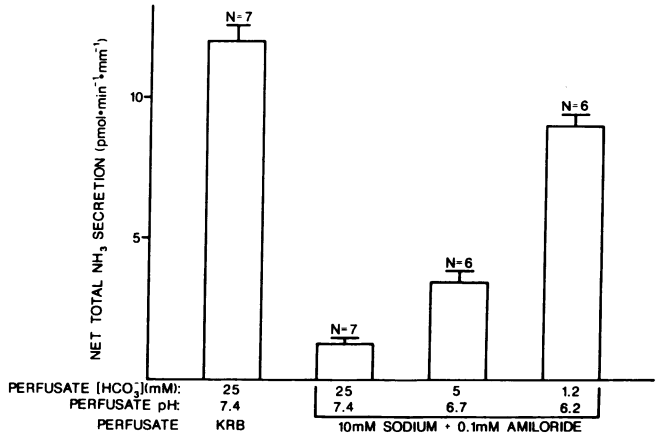

Figure 2. Net luminal secretion of total ammonia in proximal tubule segments bathed in pH 7.4 KRB buffer and perfused with KRB buffer (pH 7.4, $[HCO_3^-]$ = 25 mM) or with various modified KRB buffers containing 10 mM sodium and 0.1 mM amiloride.

of luminal fluid from tubules perfused with the modified KRB buffer containing 25 mM bicarbonate, but did not differ significantly from the pH of luminal fluid from tubules perfused with the control KRB buffer. Total ammonia production rates did not significantly differ among the four groups of tubules perfused with the various perfusates (Table I).

The net total ammonia secretion rates were measured in tubules perfused with control KRB buffer and with modified KRB buffers containing 10 mM sodium, 0.1 mM amiloride, and various concentrations of bicarbonate. Perfusion flow rates did not differ significantly among groups and averaged 21.0±1.0 nl/min. The net rates of total ammonia secretion observed in tubules perfused with the modified KRB buffers containing 10 mM sodium buffer and 0.1 mM amiloride were higher in the groups perfused with the modified KRB buffers containing 1.2 or 5 mM bicarbonate concentrations than in the group perfused with the modified KRB buffer containing 25 mM bicarbonate (Fig. 2). The net rate of total ammonia secretion of proximal tubule segments perfused with the modified KRB buffer containing 5 mM bicarbonate was 3.5±0.4 pmol/min per mm, and the net rate of secretion observed in tubule segments perfused with the modified KRB buffer containing 1.2 mM bicarbonate was 9.1±0.5 pmol/min per mm. The rates of total ammonia secretion observed in tubules perfused with 5 mM and 1.2 mM bicarbonate buffers were significantly higher than the net rate observed in tubules perfused with the modified KRB buffer containing 25 mM bicarbonate, 1.3±0.2 pmol/min per mm ($P < 0.05$ vs. the 5-mM bicarbonate group and $P < 0.01$ vs. 1.2 mM bicarbonate group), but remained lower than the rate observed in tubules perfused with the control KRB perfusate, 12.0±0.6 pmol/min per mm ($P < 0.01$). Thus lowering the bicarbonate concentration and pH of the modified KRB perfusate containing 10 mM sodium and 0.1 mM amiloride increased the net secretion of total ammonia without significantly altering the rate of total ammonia production. Nevertheless, lowering the luminal pH of the modified KRB perfusate containing 10 mM sodium and 0.1 mM amiloride from 7.4 to as low as 6.2 failed to bring the net luminal total ammonia secretory rate up to the level observed in tubules perfused with control pH 7.4 KRB buffer.

Because it is unclear whether functional luminal carbonic anhydrase is present in the segment of the mouse proximal that we studied (13), it is also unknown whether an acid disequilibrium pH is present. Such a disequilibrium pH would not be detected by measuring the pH of the collected fluid. If present, an acid disequilibrium pH would enhance the net

Table I. Total Ammonia Production Rates by Proximal Tubules Perfused with Control KRB Buffer and Modified KRB Buffers Containing 10 mM Sodium and 0.1 mM Amiloride

Group	Perfusate	*n*	Perfusate HCO_3^-	Perfusate pH	Collected fluid pH	Total ammonia production
			mM			*pmol/min per mm*
I	Control KRB buffer	7	25	7.43±0.01	7.37±0.01	20.5±0.7
II	Low Na + amiloride	6	25	7.43±0.01	7.44±0.02*	22.1±0.6
III	Low Na + amiloride	6	5	6.74±0.01§	7.33±0.01*‡	20.5±1.5
IV	Low Na + amiloride	6	1.2	6.18±0.01§	7.23±0.01§	20.4±1.9

* $P < 0.05$ vs. group I. ‡ $P < 0.01$ vs. group II. § $P < 0.001$ vs. group I and group II.

secretion of total ammonia by permitting diffusable nonionic ammonia to be trapped as ammonium ion within the acidic luminal fluid. An acid disequilibrium pH would also explain the marked reduction of net total ammonia secretion by the inhibition of luminal acidification by perfusion of the tubule lumen ith the modified KRB buffer containing 10 mM sodium and 0.1 mM amiloride as described above. We added carbonic anhydrase (bovine type 2; Sigma Chemical Co., St. Louis, MO) to the luminal perfusate (0.1 mg/cm^3) to eliminate any disequilibrium pH (14) and measured net total ammonia secretion in the presence and absence of carbonic anhydrase in the luminal perfusate (Fig. 3). Net total ammonia secretion in tubules perfused with KRB buffer containing carbonic anhydrase was 9.9±0.4 pmol/min per mm, which was 15% lower than the rate observed in control proximal tubule segments perfused at a similar flow rate with KRB buffer without carbonic anhydrase ($P < 0.01$). The total ammonia production rate in 6 tubules perfused with carbonic anhydrase was 21.5±1.3 pmol/min per mm which did not differ significantly from 7 tubules perfused with KRB control buffer without carbonic anhydrase. Thus, addition of carbonic anhydrase to the perfusate produced only a small suppression in net total ammonia secretion without affecting total ammonia production rates.

Discussion

The present study used the technique of in vitro microperfusion to measure the release of total ammonia from isolated perfused mouse proximal tubule segments. The total ammonia production rate reflected the rate at which total ammonia was released into the bath medium from both peritubular and luminal aspects of the perfused tubule segment. The net rate of luminal total ammonia secretion equaled the rate at which total ammonia left the distal end of the perfused segment in timed luminal fluid collections. The net total ammonia secretory rate reflected the difference between the rate that total ammonia entered the luminal fluid and the rate that total ammonia moved out of the luminal fluid either through cellular reentry or a paracellular pathway.

Our previous studies demonstrated that a major portion of the total ammonia produced by a perfused proximal tubule segment exits the tubule via the luminal fluid leaving the distal end of the segment (8, 9). The high rates of luminal total ammonia secretion were remarkable in that they resulted from much higher concentrations of total ammonia appearing in collected luminal fluid samples than in the surrounding bath medium with luminal fluid-to-bath total ammonia concentration ratios exceeding 200:1. Because total ammonia appeared to enter the luminal fluid at a substantial rate despite a more favorable diffusion gradient toward the bath solution, we suggested that total ammonia secretion into the luminal fluid occurred more readily than movement into the peritubular fluid. To explore further the mechanism of net luminal total ammonia secretion, we studied the effect of the inhibition of luminal Na^+-H^+ exchange on luminal total ammonia output and luminal total ammonia production.

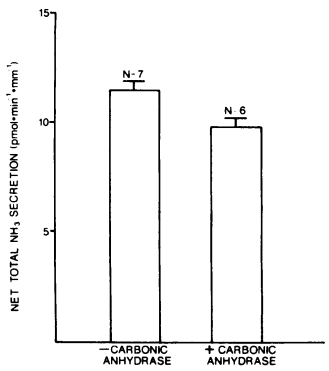

Figure 3. Net luminal secretion of total ammonia in proximal tubule segments bathed in pH 7.4 KRB buffer and perfused with pH 7.4 KRB buffer with or without carbonic anhydrase.

The results of the present study suggested an important role of the Na^+-H^+ exchanger in net luminal total ammonia secretion. Perfusion with a modified KRB buffer containing 10 mM sodium and 0.1 mM amiloride inhibited measurable acidification of the luminal fluid presumably by inhibition of Na^+-H^+ exchange. Previous studies by others in rabbit brush border membrane vesicles (15) and in the rat proximal tubule perfused in vivo (16) have demonstrated the inhibitory effect of amiloride Na^+-H^+ exchange and on fluid reabsorption. In the present study, when proximal tubule segments were perfused with the modified KRB buffer containing 10 mM sodium and 0.1 mM amiloride, the net rate of luminal total ammonia secretion was only 10% of the rate observed in tubules perfused with control KRB buffer. In contrast, the total ammonia production rate in proximal tubules perfused with the modified KRB buffer remained at control levels. Thus, the overall rate of total ammonia released by the proximal tubule was unaffected by the inhibition of the Na^+-H^+ exchanger whereas the net luminal secretion of total ammonia was markedly inhibited. Inhibition of Na^+-H^+ exchange could theoretically affect luminal total ammonia entry by two mechanisms: (*a*) inhibition of the acidification process through inhibition of Na^+-H^+ exchange which would diminish trapping of ammonia as ammonium ion (17); and (*b*) inhibition of sodium-ammonium ion exchange (18).

According to the diffusion trapping model, the movement of total ammonia across the luminal membrane would occur by diffusion of nonionic ammonia across the luminal membrane into the luminal fluid. Accumulation of total ammonia within the luminal field would be driven by an acidic luminal fluid pH which would promote the titration of nonionic ammonia to ammonium ion. Ammonium ion would be trapped within the lumen because it presumably would not diffuse out of the luminal fluid via back diffusion through the luminal membrane. To determine the relative importance of luminal acidity on luminal ammonia entry, proximal tubule segments were perfused with low sodium perfusates containing amiloride in which the pH of the perfusate was lowered in a graded fashion by lowering the bicarbonate concentration. The reduction of the pH of the low sodium perfusates containing amiloride reduced collected fluid pH compared to collected fluid pH in proximal tubules perfused with pH 7.4 KRB buffer containing 151 mM sodium without amiloride. The reduction in collected fluid pH was not as great as was previously reported by us using KRB buffer with normal sodium concentrations in which the initial bicarbonate concentration was lowered to the same extent (9). In our previous study, lowering

the bicarbonate concentration of the KRB perfusate from 25 to 5 or 1.2 mM lowered the collected fluid pH from 7.34 to 6.97 or 6.75, respectively. The higher pH values observed with perfusion with the low sodium perfusates containing amiloride compared with perfusion with the high sodium perfusates may have resulted from inhibition of luminal acidification in tubules perfused with the low sodium perfusates containing amiloride.

Net total ammonia secretory rates increased as the pH and bicarbonate concentrations were lowered in the low sodium perfusates containing amiloride while total ammonia production rates remained unchanged. Yet the net rates of total ammonia secretion did not return to the levels observed in tubules perfused with control pH 7.4 KRB buffer without amiloride even though the collected luminal fluid and average luminal fluid pH of the segments perfused with the low pH perfusates containing 10 mM sodium and 0.1 mM amiloride were lower than those observed in tubules perfused with pH 7.4 control KRB perfusate. These data demonstrated that luminal acidity by itself was not the only driving force in determining the rate of net total ammonia secretion and that the mechanism by which the inhibition of the Na^+-H^+ exchanger reduced net total ammonia secretion may be through the direct inhibition of the exchange of sodium for ammonium ions on the Na^+-H^+ exchanger (18).

An absence of carbonic anhydrase in contact with the luminal fluid could generate an acid disequilibrium pH which would result in lower intraluminal pH values than would be evident by pH measurements in collected luminal fluid samples. Such a reduced intraluminal pH would favor trapping of total ammonia within the luminal fluid as ammonium ion. It is not clear how much carbonic anhydrase is in contact with the luminal fluid of the mouse proximal tubule segments employed in the present studies although histologic studies suggest carbonic anhydrase may be present in the cells of the late convoluted and early straight portions of the mouse proximal tubule (13). Addition of carbonic anhydrase to the luminal fluid would abolish the disequilibrium pH, if present, and should reduce luminal total ammonia entry dependent upon nonionic diffusion of ammonia into the lumen and trapping of ammonium ion within the lumen (14). The present studies demonstrated only a 15% reduction in net total ammonia secretion with addition of carbonic anhydrase to the perfusate suggesting that a disequilibrium pH played only a minor role in net total ammonia secretion under these experimental conditions.

The relative contribution of sodium-ammonium ion exchange and diffusion-trapping on net total ammonia secretion may depend upon intraluminal pH. At an initial luminal pH of 7.4 and bath pH of 7.4, conditions that approximate intraluminal and peritubular pH in the very early segments of the proximal tubule, Na^+-H^+ exchanger activity would be quite active because of the large lumen-to-cell sodium gradient and cell-to-lumen hydrogen ion gradient. Under such conditions, secretion of the total ammonia made within the proximal tubule cell would be greatly facilitated by Na^+-NH_4^+ exchange but not by diffusion of nonionic ammonia with trapping of ammonium ion which requires a favorable pH gradient. At low intraluminal pH values, conditions which may be observed in the late proximal tubule, Na^+-H^+ exchanger activity may be minimal (19), but diffusion of NH_3 and trapping of NH_4^+ within the lumen may be the dominant process in luminal total ammonia secretion. Consistent with this concept are the results of micropuncture studies by Good and colleagues (20) that demonstrate the generation of relatively large total ammonia concentrations within the lumen in the earliest portions of the proximal tubule even in the absence of large lumen-to-blood pH gradients.

In conclusion, a major portion of the total ammonia produced by an isolated perfused mouse proximal tubule segment leaves the tubule via the luminal fluid. Net total ammonia secretion was markedly inhibited by perfusing proximal tubule segments with a low sodium buffer containing amiloride, and this inhibition was only partially reversed by acidifying the luminal fluid. When a proximal tubule segment is perfused with and bathed in buffers with the same pH of 7.4, the major mechanism by which the Na^+-H^+ exchanger facilitates secretion of total ammonia into the lumen may be through Na^+-NH_4^+ exchange.

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