Role of Countercurrent Multiplication in Renal Ammonium Handling: Regulation of Medullary Ammonium Accumulation[1,2]

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ABSTRACT

Ammonium (NH_3 plus NH_4^+), produced predominantly in the proximal tubule, is transferred to the final urine by a process involving countercurrent multiplication of ammonium which generates an ammonium concentration gradient in the renal medulla. It was hypothesized that if urinary ammonium excretion rates are controlled in part by the medullary ammonium gradient, changes in hydration and acid-base state should cause changes in the medullary ammonium gradient consistent with expected changes in urinary ammonium concentrations. To test that hypothesis, rats were subjected to water diuresis, water deprivation, water deprivation plus furosemide, and dietary acid and base loads and corticomedullary ammonium gradients in their kidneys were then measured. Sections were cut along the corticomedullary axis to yield slices of cortex, outer stripe of outer medulla, inner stripe of outer medulla, and three levels of the inner medulla. The total ammonia content of homogenized slices was measured by either a membrane ammonia electrode or an enzymatic technique. Kidneys from water-deprived animals showed a distinct ammonium gradient along the corticomedullary axis, with the highest contents found at the tip of the papilla. The gradient was attenuated by water diuresis and abolished by furosemide. Acid loading enhanced the gradient, and base loading abolished it. These results indicate that the corticomedullary ammonium gradient is regulated in response to changes in hydration and acid-base state.

Key Words: *Ammonia excretion, countercurrent mechanism, renal acid-base balance, renal medullary ammonia gradient*

Regulation of renal ammonium (NH_4^+ plus NH_3) production and excretion plays an important role in mammalian acid-base homeostasis, with production and excretion increasing during acidosis and decreasing during alkalosis. Ammonium is produced chiefly in cortical proximal tubules and is secreted into tubular fluid. It has been postulated that secreted ammonium is transferred to the final urine by a process which depends upon countercurrent multiplication of ammonium in the medulla which raises the medullary interstitial ammonium concentration above that of the cortex. Then, as a result of proton secretion into the collecting duct, which acidifies the lumen, urine ammonium concentration can rise above that of medullary interstitial fluid (1,2).

The view that ammonium is concentrated in the renal medulla by countercurrent multiplication originated with the studies of Robinson and Owen (3) and Sullivan (4) in 1965. Robinson and Owen (3) found high ammonium concentrations in medullary tissues from nondiuretic dogs, with the highest levels in the inner medulla. Similar observations have been made in rats (5,6). Based on stop-flow results in dogs, Sullivan (4) also came to the conclusion that ammonium accumulates in the medulla and hypothesized that the mechanism might be similar to the countercurrent multiplication of sodium. However, the energy source ("single effect") for an ammonium multiplier was unknown. About 20 years later, Good *et al.* (7) showed in rats that ammonium is reabsorbed against a concentration gradient in the thick ascending limb (TAL) of Henle's loop. They proposed that this transport process was the single effect for the countercurrent concentration of ammonium. In subsequent studies, Garvin *et al.* (8) and Good (9) demonstrated that a substantial part of the TAL ammo-

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nium transport occurs by a secondary active transport mechanism in which NH_4^+ substitutes for K^+ on the apical Na^+-K^+-$2CL^-$ cotransporter. The high ammonium concentration in the renal medulla generated by countercurrent multiplication is, to a large extent, responsible for the interstitial-to-lumen NH_3 concentration gradient across the collecting duct epithelium that drives NH_3 entry into the collecting duct lumen (10) in parallel with H^+ secretion.

If urinary ammonium excretion rates are controlled in part by the medullary interstitial ammonium gradient, it is reasonable to hypothesize that changes in acid-base state and hydration will result in modification of the gradient in a manner consistent with the expected changes in urinary ammonium concentration. We tested that hypothesis by studying the effects of dehydration, water diuresis, furosemide treatment, and dietary acid and base loads on total ammonia content of slices of rat kidney tissues sampled along the corticomedullary axis.

METHODS

Animal Treatment

Two series of experiments were conducted. In the first, male Sprague-Dawley rats weighing from 125 to 185 g were made water diuretic by being given 600 mM sucrose solution to drink overnight (N = 6). A second group of rats (N = 6) was water deprived overnight, and the third group (N = 5) was water deprived overnight and in the morning was given an i.p. injection of furosemide (5 mg/rat). Furosemide-injected animals were sampled after they spontaneously produced urine having an osmolality less than 400 mosm/kg. The time between furosemide injection and sampling ranged from 45 to 60 min.

In the second series, male Sprague-Dawley rats weighing from 205 to 272 g were placed in metabolic cages and were given food *ad libitum*. Rats were acid (N = 9) or base (N = 10) loaded by being provided with either 50 mM NH_4Cl or 50 mM $NaHCO_3$ as drinking water given *ad libitum* for 7 to 10 days before sampling. This concentration of NH_4Cl and $NaHCO_3$ was chosen so that the animals would be subjected to mild acid and base loads similar to the loads given in a previous study of rat acid-base balance in this laboratory (11) in which NH_4Cl and $NaHCO_3$ loads ranged from 8.08 to 8.39 μEq/g/day.

On the basis of the volume of fluid drunk by the rats in this study, those in the acid-loaded group ingested 9.87 ± 0.63 μEq/g/day of NH_4Cl and those in the base-loaded group ingested 9.52 ± 0.92 μEq/g/day of $NaHCO_3$. Acid- and base-loaded animals were compared with one another and with untreated controls (N = 8).

Tissue Preparation

To measure tissue ammonium content, rats were anesthetized with Nembutal (i.p. injection of approximately 5 mg/100 g) and the kidneys were removed and immediately frozen on dry ice. They were then sliced frozen to yield a column of tissue which extended from the cortex to the tip of the papilla. Sections were cut along the corticomedullary axis as shown in Figure 1 to yield six slices: cortex (COR), outer stripe of outer medulla (OS), inner stripe of outer medulla (IS), and three inner medullary sections (I-1, I-2, and I-3).

Analytical Methods

Total ammonia contents of slices were assayed by one of two different methods. In experiments on the effect of hydration state, frozen tissue slices were placed in 250 μL of cold 350 mM NaOH and homogenized. Total ammonia concentration of the homogenates was then measured by using a membrane ammonia electrode (Orion Model 95-12). After the total ammonia concentration was determined, samples of the homogenate were analyzed for protein content by the BioRad protein assay with gamma-globulin in 1 N NaOH as a standard. Total ammonium contents of the original tissue slices are expressed per milligram of protein.

In the experiments on acid and base feeding, tissue ammonium contents were measured by an enzymatic technique (Sigma Diagnostic Kit #170-UV). Tissue slices were homogenized in 0.5 mL of ice cold 7%

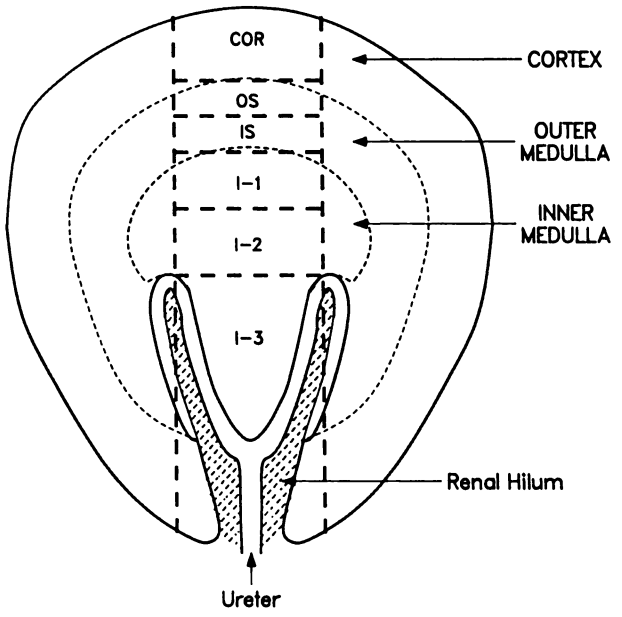

Figure 1. Medial section of the rat kidney showing slicing technique and terminology.

trichloroacetic acid, and the solution was centrifuged. The supernatant was drawn off and the pH of a 0.42-mL sample was adjusted to near neutral by the addition of 0.02 mL of 10 mM Na_2HPO_4 in 9 N NaOH. A 0.2-mL sample of buffered supernatant was then analyzed for ammonium. The pellet was resuspended in 1 N NaOH, shaken overnight, and analyzed for total protein by the BioRad protein assay as described above.

Statistical Analysis

Significant differences at alpha levels of 0.05 among means of treatment groups were determined by doing a one-way analysis of variance (ANOVA) and then using Duncan's multiple-range test. All values are reported as mean ± SE, unless otherwise noted.

RESULTS

Effects of Hydration State and Furosemide

During water diuresis, urine osmolality averaged about 200 mosm/kg, compared with an average of 1,605 mosm/kg in water-deprived animals (Table 1).

TABLE 1. Effect of hydration state and the loop diuretic, furosemide, on the renal corticomedullary ammonium concentration gradient: urine osmolality[a]

Group	osm – mosm/kg
Water diuretic	199.3 ± 29.9
Water deprived	1,604.7 ± 137.3
Water deprived plus furosemide	320.8 ± 16.7

[a] Values are mean ± SE.

A pronounced corticomedullary gradient for tissue ammonium content was found in water-deprived rats (Table 2; Figure 2). The ammonium gradient was attenuated during water diuresis but was still significant. On the other hand, treatment of water-deprived rats with furosemide abolished the ammonium gradient and, in comparison with water-deprived rats, reduced tissue ammonium in all slices.

Effects of Acid and Base Loading

Acid and base loading caused predictable changes in urine pH, ammonium concentration, and total ammonia excretion (Table 3) which were very similar to changes in the same parameters seen in an earlier study of rat acid-base balance in this laboratory (11). In acid-loaded rats, urine pH was 1.5 U lower, urine ammonium concentration was about six times higher, and total ammonia excretion was about eight times greater than in base-loaded animals. As expected, there was a strong correlation ($r^2 = 0.78$) between urinary pH and urinary ammonium concentration (Figure 3).

As can be seen in Table 4 and Figure 4, there was a marked corticomedullary ammonium content gradient in control animals which was enhanced by acid loading. In acid-loaded animals, the ammonium content of the I-2 and I-3 slices from the inner medulla was significantly higher than that in controls. Base loading abolished the ammonium gradient and the ammonium content of all slices. Even slices from the cortex and outer medulla of base-loaded animals had ammonium contents significantly lower than those in the acid-loaded group.

DISCUSSION

Previous investigators have demonstrated a distinct corticomedullary ammonium gradient in the

TABLE 2. Total ammonia contents of tissue slices[a]

Slice	Water Diuretic (DI)	Water Deprived (WD)	Water Deprived Plus Furosemide (WD + F)	Significant Differences Among Treatment Within Slices[b]
Cortex	43.6 ± 6.8[a]	43.4 ± 2.5	22.0 ± 6.0	WD + F < DI and WD
Outer stripe	46.9 ± 4.5	56.0 ± 7.4	25.1 ± 5.3	WD + F < WD
Inner stripe	55.7 ± 8.4	62.3 ± 14.1	16.6 ± 3.3	WD + F < DI and WD
Inner medullary 1	58.0 ± 27.3	110.6 ± 23.7	22.0 ± 3.3	WD + F < WD
Inner medullary 2	78.0 ± 5.3	112.6 ± 8.7	28.7 ± 5.5	WD + F < DI < WD
Inner medullary 3	79.0 ± 19.1	136.9 ± 23.1	19.5 ± 3.1	WD + F < DI and WD

[a] Values for total ammonia contents are means ± SE for 17 animals expressed as nanomoles per milligram of protein. Means within treatment groups which are adjacent to the same vertical bar are not significantly different at the 0.05 level by using one-way ANOVA and Duncan's test.

[b] This column shows significant differences among the three treatments of the total ammonia contents in slices of tissue from the same level. Significance at the 0.05 level was determined by using one-way ANOVA and Duncan's test.

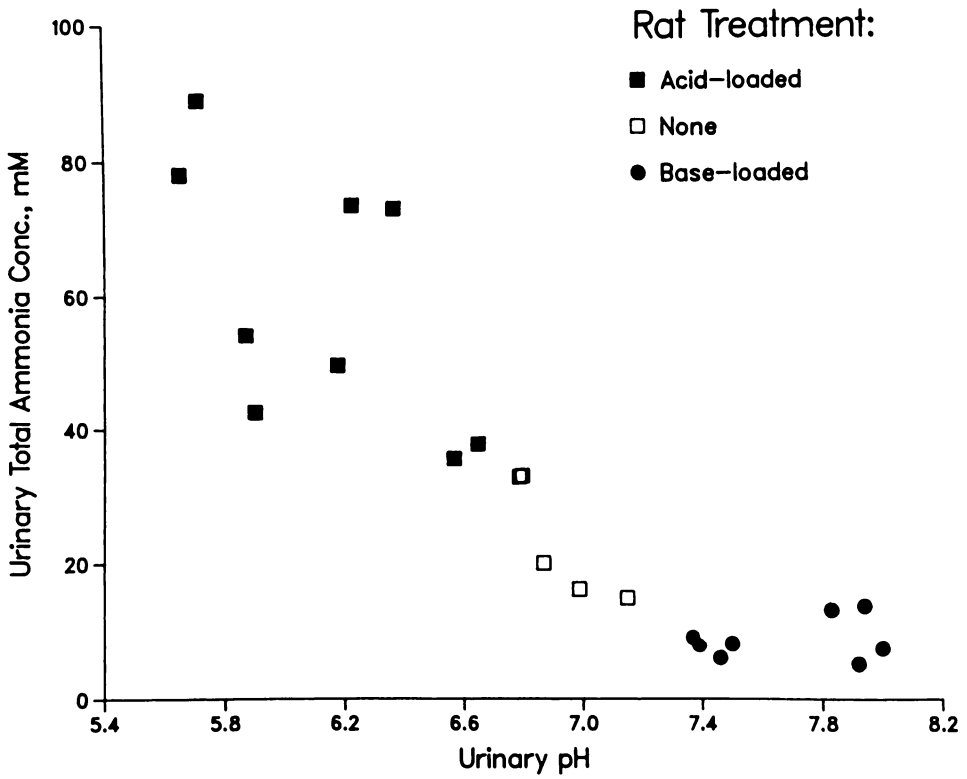

Figure 2. Relationship between urine pH and ammonium concentration. Values plotted for control animals (N = 5) are indicated by an open square, those for acid-loaded animals (N = 9) are indicated by a solid square, and those for base-loaded animals (N = 8) are indicated by a solid circle. An unweighted least-squares linear regression of ammonia concentration versus pH yielded an r^2 = 0.78.

TABLE 3. Effects of acid and base loading on urine composition and total ammonia excretion[a]

Treatment	osm – mosm/kg	pH	Ammonium (mM)	Total Ammonia Excretion (μEq/g/day)
Control (C)	1,934 ± 115 (N = 7)	7.10 ± 0.08 (N = 7)	23.4 ± 4.0 (N = 5)	1.04 ± 0.17 (N = 4)
Acid (A)	1,788 ± 59 (N = 9)	6.13 ± 0.15 (N = 9)	59.4 ± 6.5 (N = 9)	4.14 ± 0.86 (N = 4)
Base (B)	1,459 ± 119 (N = 8)	7.68 ± 0.10 (N = 8)	8.9 ± 1.1 (N = 8)	0.52 ± 0.10 (N = 4)
Significant differences among treatments[b]	none	B > A; C > A	A > C; A > B	A > C; A > B

[a] Values are means ± SE.

[b] Significant differences among treatment means at the 0.05 level were determined by one-way ANOVA and Duncan's test.

kidneys of nondiuretic rats (5,6). In this report, we have shown that the gradient can be modified significantly by changes in hydration or acid-base state, in a manner consistent with the view that the gradient is an important determinant of urinary ammonium concentration.

Water diuresis induced by providing 600 mM sucrose as drinking water significantly attenuated the gradient in comparison with water-deprived animals (Table 2; Figure 2). Although the gradient was diminished, it was not totally eliminated despite the fact that the rats were producing dilute urine (Table 1).

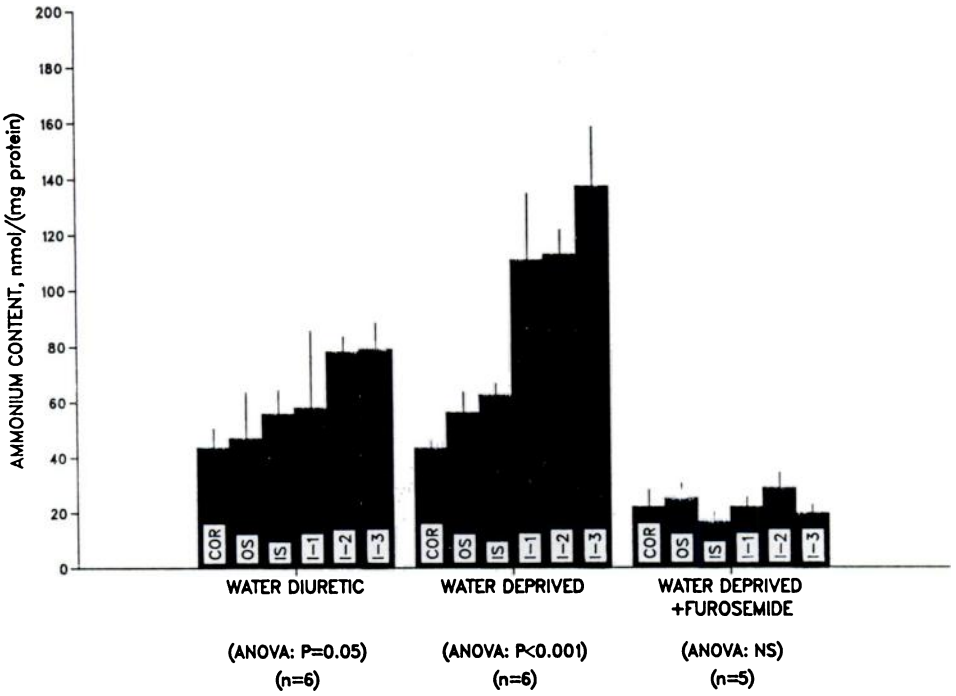

Figure 3. Ammonium content of kidney slices (nanomoles per milligram of protein) for water-diuretic, water-deprived, and water-deprived plus furosemide rats. Vertical bars indicate 1 SD about mean values. ANOVA values indicate significant differences among slices, within treatments.

TABLE 4. Effect of acid and base loading on the renal corticomedullary ammonium concentration gradient

Slice	Total Ammonium Content of Tissue Slices[a]			Significant Differences Among Treatment Within Slices[b]
	Control	Acid	Base	
Cortex	27.5 ± 3.3	40.4 ± 3.4	25.3 ± 4.6	A > B
Outer stripe	31.7 ± 4.0	44.4 ± 6.3	24.3 ± 4.1	A > B
Inner stripe	30.5 ± 1.2	35.8 ± 3.51	21.5 ± 2.7	A > B
Inner medullary 1	75.2 ± 7.9	91.0 ± 11.8	29.0 ± 4.7	A > B, C > B
Inner medullary 2	85.5 ± 5.1	171.6 ± 10.8	30.8 ± 4.5	A > C > B
Inner medullary 3	108.5 ± 14.3	176.2 ± 9.8	40.4 ± 5.8	A > C > B

[a] Values for total ammonia contents are means ± SE for 15 animals expressed as nanomoles per milligram of protein. Means within treatment groups which are adjacent to the same vertical bar are not significantly different at the 0.05 level by one-way ANOVA and Duncan's test.

[b] This column shows significant differences among the three treatments of the total ammonia content slices of tissue from the same level. A, acid loaded; B, base loaded; and C, control. Significance at the 0.05 level was determined by one-way ANOVA and Duncan's test.

This suggests that even during water diuresis, countercurrent multiplication of ammonium persists, creating an interstitium-to-lumen NH_3 gradient that could drive NH_3 secretion into the medullary collecting duct. It is not surprising that countercurrent multiplication of ammonium persists during water diuresis because it has been shown that the rate of active NH_4^+ transport in the TAL is not affected by vasopressin (12).

These results are in accord with our current knowledge of the countercurrent mechanism. In its simplest terms, the concentrating mechanism can be thought of as a means of varying water excretion without obligatory changes in solute excretion, thus allowing water and solute excretion to be regulated independently (13). In this simple model, urinary solute concentration varies inversely with the urinary flow rate so that the product of those two vari-

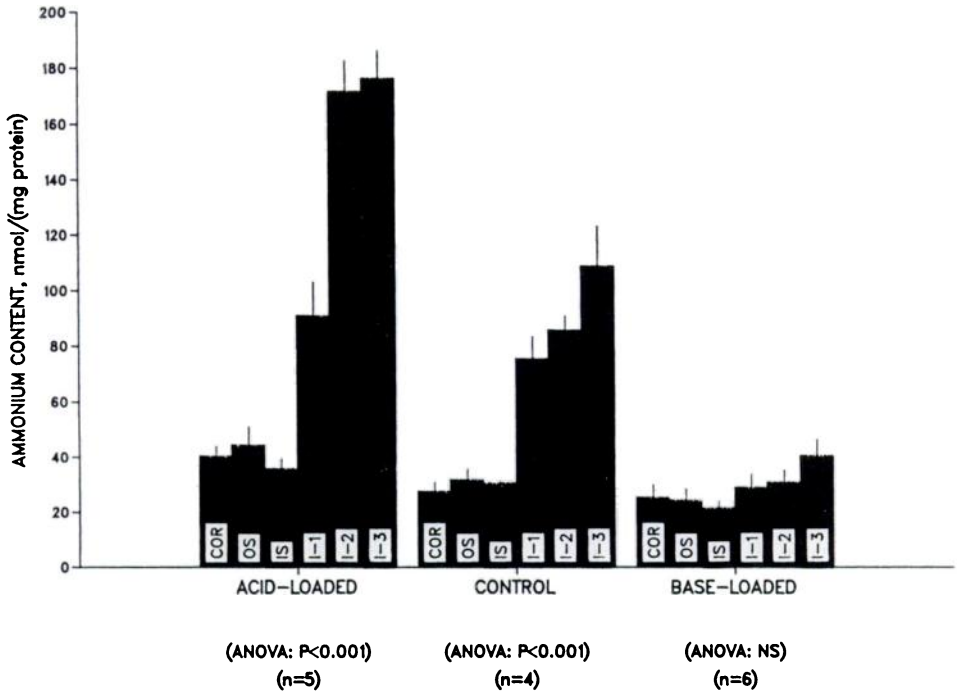

Figure 4. Ammonium content of kidney slices (nanomoles per milligram of protein) for acid-loaded, control, and base-loaded rats. Vertical bars indicate 1 SD about mean values. ANOVA values indicate significant differences among slices, within treatments.

ables (the solute excretion rate) remains constant. For a number of solutes (such as urea), a major determinant of the urinary concentration is the concentration in the medullary interstitium. We propose that this is true for ammonium as well. Specifically, we suggest that the fall in ammonium content of the medullary tissue contributes to a decrease in urinary ammonium concentration and consequently tends to maintain a steady level of ammonium excretion despite a marked increase in urinary volume flow rate.

It seems pertinent to ask in the present context what advantage is gained by short-circuiting ammonium from the loop of Henle to the collecting ducts in the renal medulla. Theoretically, it could be argued that ammonium excretion could be maintained constant despite changes in urine flow if ammonium was secreted in the proximal tubule and simply stayed in the tubule lumen in subsequent segments. We suggest, however, that water absorption in the late distal tubule and cortical collecting duct causes NH_3 backflux from lumen to blood as a result of three effects: (*1*) water absorption concentrates NH_3 and NH_4^+ in the lumen generating large transepithelial concentration gradients; (*2*) water absorption slows luminal flow rates, thus increasing contact times for diffusion; and (*3*) water absorption may cause solvent drag of NH_3. Although NH_3 permeabilities of some renal tubule segments may be very low (8,14), an extremely low NH_3 permeability may be physically incompatible with the requirement for a high water permeability in collecting ducts. Thus, we propose that short-circuiting of ammonium from the medullary loops to the medullary collecting ducts may be a means of maintaining a low luminal total ammonium concentration in the distal tubule and cortical collecting duct to minimize NH_3 loss from the lumens, while continuing to excrete ammonium at high concentrations.

Abolition of the ammonium gradient by furosemide, an inhibitor of active NH_4^+ absorption in the TAL (Table 2; Figure 2), is consistent with the hypothesis that the generation of the gradient is dependent on active absorption of NH_4^+ by the TAL. Furosemide directly blocks the apical Na^+-K^+-$2Cl^-$ cotransporter on which NH_4^+ can substitute for K^+ (8,9,15). Although furosemide abolished the corticomedullary ammonium gradient, it is clear from other studies that furosemide and other loop diuretics do not depress ammonium excretion when given acutely and, in some cases, increases ammonium excretion mildly. These results therefore indicate that ammonium excretion can be maintained in the absence of medullary ammonium accumulation. We speculate, however, that the maintenance of ammonium excretion under these circumstances is dependent on the rapid tubule fluid flow rates through the distal tubule and cortical collecting duct induced by loop diuretics. The rapid flows would be expected to minimize con-

tact time of the tubular fluid with the tubular epithelium and limit back diffusion of NH_3 from lumen to blood.

If regulation of countercurrent multiplication of ammonium is involved in overall regulation of ammonium excretion, one would expect the corticomedullary ammonium gradient to vary with acid and base loading. In our experiments, acid loading intensified the gradient in comparison with controls and base loading abolished it (Table 4; Figure 4). This would explain how the corticomedullary ammonium concentration gradient, working in conjunction with diffusion trapping in the medullary collecting duct, would allow ammonium excretion rates to be high in response to an acid load even when the animal is in a state of antidiuresis and urine volume is low.

The changes in the corticomedullary ammonium gradient with changes in acid-base intake could have resulted from changes in ammonium transport by the loop of Henle or simply from changes in the delivery of ammonium to the loop of Henle from the proximal tubule. It is widely accepted that the chief site of regulation of ammonium production in the kidney is the proximal tubule (2). Furthermore, it has been demonstrated in isolated rat tubule segments that glutamine-dependent ammonium production is altered in response to changes in acid-base intake in a manner which parallels the changes in medullary ammonium accumulation shown in Figure 4. Consequently, we favor the view that ammonium accumulation in the rat renal medulla is regulated principally through regulation of ammonium production in the proximal tubule, which affects the rate at which ammonium is supplied to the countercurrent multiplier.

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