Gamble’s “economy of water” revisited: studies in urea transporter knockout mice

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Fenton, Robert A., Chung-Lin Chou, Holly Sowersby, Craig P. Smith, and Mark A. Knepper. Gamble’s “economy of water” revisited: studies in urea transporter knockout mice. Am J Physiol Renal Physiol 291: F148–F154, 2006. First published February 14, 2006; doi:10.1152/ajprenal.00348.2005.—The Gamble phenomenon (initially described over 70 years ago as “an economy of water in renal function referable to urea”) suggested that urea plays a special role in the urinary concentrating mechanism and that the concentrating mechanism depends in some complex way on an interaction between NaCl and urea. In this study, the role of collecting duct urea transporters in the Gamble phenomenon was investigated in wild-type mice and mice in which the inner medulla collecting duct (IMCD) facilitative urea transporters, UT-A1 and UT-A3, had been deleted (UT-A1/3−/−mice). The general features of the Gamble phenomenon were confirmed in wild-type mice, namely 1) the water requirement for the excretion of urea is less than for the excretion of an osmotically equivalent amount of NaCl, and 2) when fed various mixtures of urea and salt in the diet, less water is required for the excretion of the two substances together than the amount of water needed for the excretion of the two substances separately. In UT-A1/3−/−mice, the amount of water needed for the excretion of urea is less than for the excretion of an osmotically equivalent amount of NaCl.

METHODS

The experiments described in this manuscript are based on those initially performed in rats by Gamble et al. (8, 9). All animal studies in this paper were done under National Institutes of Health Animal Care and Use Committee-approved animal protocols (1-KE-1, 1-KE-2, 2-KE-1, H-0045, H-0047).

Diet. The basal diet consisted of a gelled diet made up of (per 5 g total) 1 ml of deionized water, 4 g of special low-salt (NaCl) synthetic food [0.001% NaCl (wt/wt); Research Diets (New Brunswick, NJ)], and 25 mg of agar; the basal diet contained 4% protein by weight (as casein). As established previously (6), the basal diet was determined to be sufficient for nutritional maintenance throughout the duration of experiments in both wild-type and UT-A1/3−/−mice.
the study. The aim of the initial experiment was to measure the concentrations of NaCl and urea in the urine when these substances were added to the basal diet in isosmotic quantities, either singly or as a mixture. Thus for the first experimental period, urea alone was added to the diet. Then, in successive periods the urea was progressively replaced by NaCl (see Table 1), until the final period when it was replaced entirely. In all experiments, the total number of osmotically active particles added to the diet remained the same, 1.5 mosmol/g of food.

Initial metabolic cage study. Six male UT-A1/3−/− mice and 6 wild-type mice of the same background (C57BL/6J) and age (14 wk) were used for all studies. The studies were continuous; thus each animal served as its own control for each dietary condition. Mice were maintained in metabolic cages for the duration of the study, under controlled temperature and light conditions (12:12-h light-dark cycles). Initially, all mice received a fixed daily ration of 5 g of gelled diet containing either urea, NaCl, or a mixture of both (see above). Mice had access to supplemental drinking water throughout the duration of the study. After 3 days of adaptation to the diet, the mice were switched to clean metabolic cages and urine was collected under mineral oil in preweighed collection vials for two successive 24-h periods and analyzed. Mice were then fed the next diet in the series, and the same regime was repeated.

Second metabolic cage study. Four male UT-A1/3−/− mice and 4 wild-type mice of the same background (C57BL/6J) and age (12 wk) were used for all studies. Studies were performed as outlined above, except the diets were as follows: diet 1, 1.5 mosmol NaCl/g food; diet 2, 1.0 mosmol NaCl/g food; and diet 3, 1.0 mosmol NaCl/g food plus 0.5 mosmol urea/g food. Mice had access to supplemental drinking water throughout the duration of the study.

Third metabolic cage study. Five male C57BL/6J wild-type mice 12 wk of age were housed in metabolic cages as outlined above. Mice received a fixed daily ration of 5 g of gelled diet containing 0, 1.0, 1.5, or 2.0 mosmol urea/g food or NaCl. Mice had access to supplemental drinking water throughout the duration of the study.

Urine analysis. Urine volume was measured gravimetrically assuming a density of one. Urine osmolality was determined using a vapor pressure osmometer (Wescor). Na+, urea, and K+ concentrations in the urine were determined using an autoanalyzer (Monarch 2000, Instrumentation Laboratories). Osmolality due to non-urea and non-NaCl solutes was calculated as follows: osmolality = total osmolality − [(urea × 0.96) + ([Na] × 1.84)], where 0.96 and 1.84 are the osmotic coefficients for urea and Na, respectively (3), and brackets indicate concentration.

Table 1. Content of diets in initial metabolic cage study

<table>
<thead>
<tr>
<th>Diet No.</th>
<th>Substance Added to Basal Diet</th>
<th>Single, mosmol/g food</th>
<th>Total, mosmol/g food</th>
<th>Substance Added, mg/g food</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Urea 1.5</td>
<td>1.5</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>NaCl 1.2</td>
<td>1.5</td>
<td>72</td>
<td>8.7</td>
</tr>
<tr>
<td>3</td>
<td>Urea 1.0</td>
<td>1.0</td>
<td>60</td>
<td>14.5</td>
</tr>
<tr>
<td>4</td>
<td>NaCl 0.5</td>
<td>0.5</td>
<td>45</td>
<td>21.75</td>
</tr>
<tr>
<td>5</td>
<td>Urea 0.5</td>
<td>0.5</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>6</td>
<td>NaCl 1.0</td>
<td>1.0</td>
<td>29</td>
<td>45.5</td>
</tr>
</tbody>
</table>

The basal diet contained 4% protein by weight (as casein). NaCl and urea were added to the basal diet in isosmotic quantities, either singly or as a mixture. Thus, initially urea alone was added to the diet; then, the urea was progressively replaced by NaCl, until the final period when it was replaced entirely. In all experiments, the total number of osmotically active particles added to the diet remained the same, 1.5 mosmol/g food.

Statistical analysis. Unless otherwise indicated, all values are quoted as means ± SE. Initial statistical analysis was performed by ANOVA. Contrasts between a dietary condition and the previous dietary condition in the same group were done by Bonferroni’s method. P values < 0.025 were considered significant. Significant differences between two different groups of animals (knockout vs. wild-type) on the same dietary intake were made with the Student’s unpaired t-test. P values < 0.05 were considered significant.

RESULTS

In the studies performed by Gamble et al. (8, 9) in rats, the levels of urea and NaCl in the diet were varied inversely, while the total osmolar intake was kept constant. In the initial part of this study, we adopted a similar approach in both wild-type and UT-A1/3−/− mice, producing the results described below.

Figure 1 shows water excretion (Fig. 1A), urinary osmolality (Fig. 1B), and osmolar excretion (Fig. 1C). Both cardinal observations made by Gamble et al. in rats were confirmed in wild-type mice (solid lines in Fig. 1). 1) The water requirement for the excretion of ~6.6 mmol urea/day (4.7 ml/day, Fig. 1A, left) was much less than the requirement for the excretion of an equivalent osmolar quantity of NaCl (9.9 ml/day, Fig. 1A, right). 2) When mixtures of urea and NaCl were given in the diet, urinary osmolality was increased and water excretion was decreased relative to levels seen for the excretion of the osmolar equivalent amounts of either urea or NaCl alone. In agreement with the Gamble study, a maximal urinary osmolality was seen at a urea:NaCl osmolar ratio somewhat > 1 (Fig. 1B).

Contrasting results were found in UT-A1/3−/− mice (dashed lines in Fig. 1). First, the amount of water needed to excrete ~6.6 mmol urea/day was virtually identical to that needed to excrete an osmotically equivalent amount of NaCl and was substantially greater than the water excretion seen in the wild-type mice (Fig. 1A). Second, the ability of the kidneys to increase urinary osmolality to a higher level with mixtures of urea and NaCl, compared with urea or NaCl alone added to the diet, was not seen in UT-A1/3−/− mice (Fig. 1B). Thus Gamble’s “economy of water referable to urea” is dependent on facilitated urea transport in the IMCD by UT-A1, UT-A3, or both.

The excretion of urea in the same experiments is shown in Fig. 2. Urea excretion was fixed by dietary intake and therefore was virtually identical in wild-type (solid lines) and UT-A1/3−/− mice (dashed lines) (Fig. 2A). The reduction in daily urea excretion in both groups as one traverses from the left-hand side to the right-hand side of the graph reflects the replacement of urea in the diet with NaCl. In contrast, urinary urea concentration was much lower in UT-A1/3−/− mice than in wild-type mice (Fig. 2B), reflecting the higher water excretion, presumably due to the urea-induced osmotic diuresis seen in the UT-A1/3−/− mice. However, urine urea concentration decreases monotonically with reduced dietary urea intake in both groups.

The excretion of Na+ in the same experiments is shown in Fig. 3. Na+ excretion was fixed by dietary intake and therefore was virtually identical in wild-type and UT-A1/3−/− mice (Fig. 3A). The increase in daily Na+ excretion in both groups in moving from the left-hand side to the right-hand side of the graph reflects the replacement of urea in the diet with NaCl. At high urine intakes (except when no NaCl was added to the diet),
the Na\(^+\) concentration was lower in UT-A1/3\(^{-/-}\) mice than in wild-type mice (Fig. 3B), reflecting the higher water excretion, presumably due to a urea-induced osmotic diuresis. In the wild-type mice, the Na\(^+\) concentration in the urine reached a maximum with a mixture of urea and NaCl in the diet relative to that seen with NaCl alone, a behavior that contributes substantially to the observation that urine osmolality is higher with urea/NaCl mixtures than with pure NaCl.

The excretion of K\(^+\) in the same experiments is shown in Fig. 4. As for the other solutes, K\(^+\) excretion was fixed by dietary intake and therefore was virtually identical in both wild-type and UT-A1/3\(^{-/-}\) mice (Fig. 4A). However, unlike the effects seen with urea and NaCl excretion, the level of K\(^+\) excretion remained constant throughout the experiment, reflecting the constant dietary intake of K\(^+\) despite different urea/NaCl intakes. K\(^+\) concentration was lower in UT-A1/3\(^{-/-}\) mice compared with wild-type mice (Fig. 4B), again reflecting the higher water excretion due to urea-induced osmotic diuresis. In wild-type mice, K\(^+\) concentration in urine reached a maximum with a mixture of urea and NaCl in the diet compared with that seen with NaCl alone, a behavior that contributes substantially to the observation that urine osmolality is higher with urea/NaCl mixtures than with pure NaCl.

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The excretion of K\(^+\) in the same experiments is shown in Fig. 4. As for the other solutes, K\(^+\) excretion was fixed by dietary intake and therefore was virtually identical in both wild-type and UT-A1/3\(^{-/-}\) mice (Fig. 4A). However, unlike the effects seen with urea and NaCl excretion, the level of K\(^+\) excretion remained constant throughout the experiment, reflecting the constant dietary intake of K\(^+\) despite different urea/NaCl intakes. K\(^+\) concentration was lower in UT-A1/3\(^{-/-}\) mice compared with wild-type mice (Fig. 4B), again reflecting the higher water excretion due to urea-induced osmotic diuresis. In wild-type mice, K\(^+\) concentration in urine reached a maximum with a mixture of urea and NaCl in the diet compared with that seen with NaCl alone, a behavior that contributes substantially to the observation that urine osmolality is higher with urea/NaCl mixtures than with pure NaCl.
The urine osmolality due to solutes other than urea and NaCl in the same experiments are shown in Fig. 5. This variable was obtained by subtracting the osmolality due to urea (urea concentration multiplied by its osmotic coefficient of 0.96) and the osmolality due to NaCl (Na\(^+\) concentration multiplied by its osmotic coefficient of 1.84) from total urinary osmolality. This variable takes into account not only potassium salts but all other substances in urine as well. As was the case for K\(^+\), however, in wild-type mice the concentration of non-urea/non-NaCl solutes in the urine reached a maximum with a mixture of urea and NaCl in the diet. The maximum non-urea/non-NaCl solute concentration was at the same urea:NaCl ratio as that seen for a maximal urine osmolality (compare with Fig. 1B) and maximal urinary K\(^+\) concentration (compare with Fig. 4B). In contrast, in UT-A1/3\(^{-/-}\) mice, the osmolality due to non-urea/non-NaCl solutes was not significantly different throughout the range of dietary conditions.

Data in Fig. 1A show that in wild-type mice there was a striking decrease in water excretion when treatment was changed from 1.5 to 1.0 mosmol NaCl/g food and 0.5 mosmol urea/g food. Is this result due to a decrease in NaCl in the urine or the increased urea in the urine? To address this question, we did an additional metabolic cage experiment. Mice were fed a

**Fig. 3.** Daily urinary Na\(^+\) excretion (A) and urinary Na\(^+\) concentration (B) from both wild-type (■, solid lines) and UT-A1/3\(^{-/-}\) (●, dashed lines) mice. #Statistically significant difference within an animal group compared with the previous dietary condition (P < 0.025). *Statistically significant difference between wild-type and UT-A1/3\(^{-/-}\) mice on any particular diet (P < 0.05).

**Fig. 4.** Daily urinary K\(^+\) excretion (A) and urinary K\(^+\) concentration (B) from both wild-type (■, solid lines) and UT-A1/3\(^{-/-}\) (●, dashed lines) mice. #Statistically significant difference within an animal group compared with the previous dietary condition (P < 0.025). *Statistically significant difference between wild-type and UT-A1/3\(^{-/-}\) mice on any particular diet (P < 0.05).

**Fig. 5.** Urine osmolality due to non-urea and non-NaCl solutes (NUNN). The calculation was done as follows: NUNN = total osmolality – ([urea] × 0.96) + ([Na] × 1.84), where 0.96 and 1.84 are the osmotic coefficients for urea and NaCl, respectively, and brackets indicate concentration. Wild-type, ■ and solid lines; UT-A1/3\(^{-/-}\), ● and dashed lines. #Statistically significant difference within an animal group compared with the previous dietary condition (P < 0.025). *Statistically significant difference between wild-type and UT-A1/3\(^{-/-}\) mice on any particular diet (P < 0.05).
diet containing 1.5 mosmol NaCl/g food, and the urine was analyzed. The mice were then switched to a diet containing 1.0 mosmol NaCl/g food, but unlike previously, no urea was added to replace the osmotic effect of the reduced NaCl. Finally, mice were switched to a diet containing 1.0 mosmol NaCl/g food plus 0.5 mosmol urea/g food. The urine output (A), urine osmolality (B), and osmolar excretion (C) of wild-type and UT-A1/3−/− mice are shown in Fig. 6. In both groups of animals, removing NaCl from the diet (without replacing with urea) resulted in a decrease in urine volume. However, when supplemental urea was added at 0.5 mosmol/g food, no increase was seen in wild-type mice. This result indicates that the reduction in urine volume in going from 1.5 to 1.0 mosmol NaCl/g food plus 0.5 mosmol urea/g food in wild-type mice (Fig. 1A) is due solely to the reduction of NaCl rather than the addition of urea per se, confirming that the behavior is due to osmotic diuresis associated with a high level of NaCl in the urine.

Data in Fig. 1A also show that in wild-type mice there was a significant decrease in water excretion when treatment switched from 1.5 to 1.2 mosmol urea/g food and 0.3 mosmol NaCl/g food. In light of these results, it raises the possibility that >1.5 mosmol urea/g food is sufficient to result in some degree of osmotic diuresis. To address this question, we did an additional metabolic cage experiment in which we progressively increased the amount of NaCl alone or urea alone added to the diet (Fig. 7). As previously determined, increasing amounts of NaCl above 1.0 mosmol/g food obligated increased levels of water excretion. In addition, urea intakes >1.5 mosmol/g food also resulted in higher water excretion. This result suggests that although collecting duct urea transporters maintain rapid transport of urea across the IMCD epithelium, the capacity of this transport process can be exceeded. Thus with large amounts of urea excretion (>6,000 μosmol/day), urea can function as an osmotic diuretic.

**DISCUSSION**

Our understanding of renal physiology has accelerated in recent years as a result of the advent of the era of “molecular physiology,” characterized by the development and application of tools to study proteins that can mediate renal function. An example, cited at the beginning of this paper, was the cloning of urea transporter cDNAs and genes that led to development of antibodies and other tools for studying the urea transporter proteins. However, success in developing these molecular tools was highly dependent on studies that established a detailed understanding of renal physiology at a “phenomenological” level. Thus physiologists seeking to clone cDNAs, which encoded proteins with a particular role in the kidney, knew precisely what to look for because of a rich body of descriptive physiological observations made over decades. Nevertheless, there are many premolecular “loose ends,” i.e., observations made in the premolecular era of renal physiology that have greatly influenced the way we teach kidney physiology, that are not yet fully understood at a molecular level. One such loose end is Gamble’s “economy of water in renal function referable to urea.” It is imperative that renal physiologists readdress such observations with the new molecular tools that are now available.
Recently, we generated UT-A1/3 knockout mice (UT-A1/3−/−) in which the facilitative urea transporters expressed in the IMCD were deleted. In contrast to wild-type mice, the IMCDs from UT-A1/3−/− mice have a very low urea permeability that does not increase with vasopressin, and these mice excrete much more water, unless the rate of urea excretion is diminished by dietary protein restriction. We concluded that in the absence of the urea transporters that are normally expressed in the IMCD, water excretion is increased as a result of urea-dependent osmotic diuresis (6, 7). Furthermore, UT-A1/3−/− mice failed to accumulate urea in their inner medullas, but the accumulation of NaCl was not attenuated. Thus the absence of IMCD urea transport does not prevent the concentration of NaCl in the inner medulla, contrary to what would be predicted from passive concentrating models (12, 16). These findings in UT-A1/3−/− mice added to the substantial volume of evidence from other studies (10, 11, 13) that the passive model in the form proposed by Kokko and Rector (12) and Stephenson (16) is not the explanation for the accumulation of NaCl in the renal inner medulla.

The observations by Gamble and colleagues (8), summarized at the beginning of this paper and confirmed in wild-type mice in this study, provided early evidence for a special role of urea in the renal handling of water. Indeed, the appeal of the passive model derived, in part, from the fact that it appeared to provide a plausible explanation for the Gamble findings. If the special role of urea is not attributable to an involvement of urea in the passive concentration of NaCl in the inner medulla, then what is the special role? Our studies show that the Gamble phenomenon (namely, higher urinary osmolality with urea supplementation of the diet rather than with NaCl supplementation and also higher urinary osmolality with mixtures of urea and NaCl added to the diet than with either NaCl or urea alone) is not seen in UT-A1/3−/− mice. Our data suggest that the Gamble phenomenon is largely a manifestation of facilitated urea transport in the IMCD by UT-A1 and UT-A3.

The first element of the Gamble phenomenon (a higher urinary osmolality with urea supplementation of the diet than with NaCl supplementation) can be explained directly by what is known about the role of UT-A1 and UT-A3 in the IMCD. These transporters allow rapid passive reabsorption of urea from the IMCD and accumulation of urea in the inner medullary interstitium. The high concentration of urea in the interstitium osmotically balances urea in the IMCD lumen, allowing large amounts of urea to be excreted without obligating water (1). In contrast, excretion of equivalent amounts of NaCl causes an osmotic diuresis, increasing water excretion through its osmotic effect.

Although our results clearly support a connection between IMCD urea transporters and the second element of the Gamble phenomenon (namely, higher urinary osmolality with mixtures of urea and NaCl added to the diet than with either NaCl or urea alone), it cannot be explained so readily from what is known about the role of UT-A1 and UT-A3 in the IMCD. However, one explanation can be derived from a consideration of the two ends of the Gamble water excretion curve (Fig. 1A). At the high-urea end, we speculated that the increased water excretion with greater urea excretion is owing to saturation of the urea transporters in the IMCD. Previous reports have shown that urea transport across the IMCD is indeed a saturable phenomenon, as expected from the properties of carrier proteins in general (2). In this study, we determined that saturation of IMCD urea transporters in vivo results in osmotic diuresis (Fig. 7). Thus as urea saturates the transporters, the osmotic effect of urea in the lumen will increase, resulting in a higher rate of water excretion. At the high-NaCl end of the Gamble water excretion curve (Fig. 1A), we propose that the increase in water excretion with a greater NaCl intake can be

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**Fig. 7.** Wild-type mice were housed in metabolic cages and initially received a 4% protein diet. The diet was sequentially supplemented with either urea or NaCl (values represent substance added to diet in mosmol/g food). Daily urine volume (A), urine osmolality (B), and daily osmolar excretion (C) are shown.

A: Urine Volume (ml/day)

<table>
<thead>
<tr>
<th>Substance added to basal diet (mosmol/g food)</th>
<th>NaCl</th>
<th>Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>4.0</td>
<td>4.0</td>
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<tr>
<td>0.5</td>
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<td>5.5</td>
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</tr>
<tr>
<td>2.0</td>
<td>6.0</td>
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B: Urine Osmolality (mosmol/kg H2O)

<table>
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<th>Substance added to basal diet (mosmol/g food)</th>
<th>NaCl</th>
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</tr>
</thead>
<tbody>
<tr>
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<tr>
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<td>400</td>
</tr>
<tr>
<td>2.0</td>
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</table>

C: Osmolar Excretion (µOs/ml/day)

<table>
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<th>Substance added to basal diet (mosmol/g food)</th>
<th>NaCl</th>
<th>Urea</th>
</tr>
</thead>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>5000</td>
<td>5000</td>
</tr>
</tbody>
</table>

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One such tool is the development of mouse models, in which a single gene, or part of a gene, has been “knocked out.” Recently, we generated UT-A1/3−/− mice, in which the facilitative urea transporters expressed in the IMCD were deleted. In contrast to wild-type mice, the IMCDs from UT-A1/3−/− mice have a very low urea permeability that does not increase with vasopressin, and these mice excrete much more water, unless the rate of urea excretion is diminished by dietary protein restriction. We concluded that in the absence of the
explained by the urine NaCl concentration reaching a maximum above which NaCl concentration cannot be raised further. In the present study, the average maximum NaCl concentration is ∼420 mM, after which the addition of more NaCl in the diet results in an increase in urine output. Indeed, when a small amount of NaCl was removed from the diet (and not replaced with urea), there was a reduction in urine volume that was not affected by the addition of urea, suggesting that the NaCl concentration itself in the urine had reached a maximum (Fig. 6A), essentially due to saturation of NaCl-absorptive mechanisms along the nephron. Indeed, direct titration studies in which dietary NaCl alone was progressively increased until a maximum urinary NaCl concentration was reached (Fig. 7) showed that above daily excretion rates of 3,500 μmol, NaCl excretion could only occur via increases in water excretion, i.e., by osmotic diuresis.

Another possible explanation can be drawn from considering the regulation of thirst and drinking behavior. In our study, water intake was on an ad libitum basis. Thus any variations in water excretion were paralleled by variations in water intake. In general, differences in water intake are driven by differences in thirst. As originally described by Verney (17), NaCl and other salts are effective solutes with regard to their ability to stimulate hypothalamic osmoreceptors, whereas urea is ineffective. The stimulation of thirst by rising osmolality is a threshold phenomenon, requiring an increase in plasma osmolality above a threshold value as seen also for the regulation of vasopressin secretion. Thus one might expect that as NaCl is added to the diet, thirst would increase, although possibly only at the highest levels of salt intake. Indeed, we observed in wild-type mice that increasing NaCl intake from 1.0 to 1.5 mosmol/g food (Fig. 1), there was a marked increase in water excretion. Thus the second element of the Gamble phenomenon may be dependent on three factors: saturation of UT-A-mediated urea transport in the IMCD (at the high-urea end of the curve), saturation of NaCl absorption along the nephron (at the low-urea end), and enhancement of thirst (at the low-urea end). In general, the overall data are consistent with the view that the decreased water excretion with mixtures of urea and NaCl added to the diet is due to the separate abilities of urea and NaCl to induce osmotic diuresis at high concentrations, rather than to any specific interaction of urea transport and NaCl transport at an epithelial level.

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