Supplement 1. HPLC measurement of amiloride concentration in urine and plasma

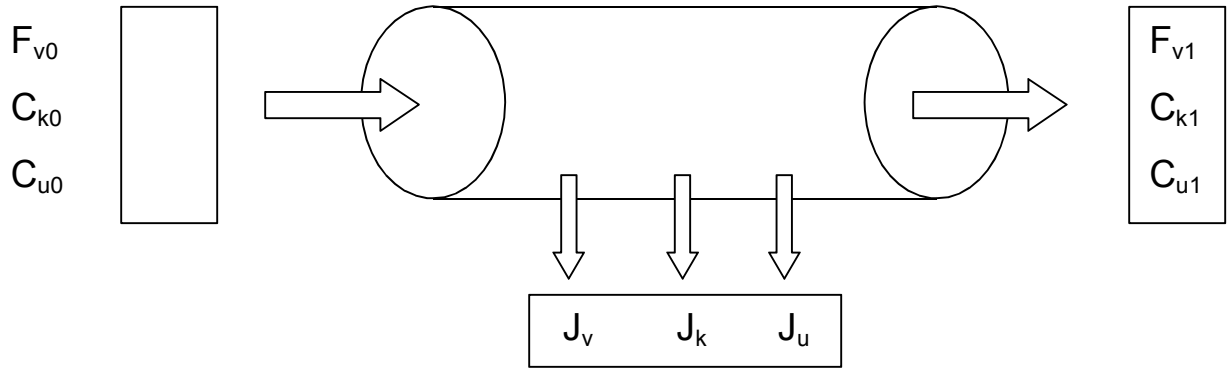
Plasma and urine amiloride concentrations were measured using high-performance liquid chromatography (HPLC). The instrument (Agilent LC 1200; Santa Clara, CA) was connected to a G1321A Agilent fluorescent detector and the chromatograms were recorded using Agilent ChemStation software. The reversed-phase separations were performed by means of a C_{18} column (Waters Spherisorb 10.0 □m) as the stationary phase. The mobile phase consisted of 20 mM ammonium acetate, pH 5.4 and acetonitrile pumped at a constant flow-rate of 1 ml/min**.** The retention time of amiloride was 18.5 min and was separated from the rest of the compounds present in urine and plasma in chromatographs obtained at 360 nm.

The time course of the concentration of amiloride in the lumen of the CNT was calculated by measuring the urine [amiloride], urine osmolality and plasma osmolality of 5 different mice of similar weight (21 – 23 gm) after 2, 4, 6, 8 and 12 hrs of treatment using the formula: urine [amiloride] X plasma [Osm]/ urine [Osm].

The trace of a urinary amiloride peak, measured 2 hrs after injection is shown in figure S1A. Figure S1B shows the plasma amiloride, 6 hrs, after injection; the area under the curve is 2.81. The scatter plot of figure S2C shows the luminal amiloride concentrations, calculated from the urine and plasma osmolalities. The bolus of amiloride (5 mg/kg) achieved a concentration of 60 μM in the terminal CCD after 2 hrs with a decline to 20 μM after 12 hrs (time of sacrifice). Figure S1D shows that the plasma amiloride concentration reaches 3 μM after 2 hrs. and gradually declines to 1.4 μM after 12 hrs. Amiloride is an effective blocker of epithelial Na channels (ENaC) in mammals, with a Ki of 100 nM. Thus, the amiloride concentration is at levels that will completely inhibit ENaC-mediated Na reabsorption in the distal nephron for the 12 hour duration.

Supplement 2. Mathematical Justification for TTKG

Impact of potassium and urea reabsorption on the TTKG



Consider a tubule with a flowing solution of two solutes, nominally KCl (k) and urea (u), in which inlet and outlet flow (F_V) and concentrations (C) are denoted by subscripts 0 and 1, respectively. Along the tubule, there is volume and solute reabsorption, J. Then mass conservation for KCl and urea are written

$$\mathbf{F_{v0}\, C_{k0} - J_k = F_{v1}\, C_{k1}}$$

$$\mathbf{F_{v0}\, C_{u0} - J_u = F_{v1}\, C_{u1}}$$

For this configuration, the osmolality, $C_T = C_k + C_u$, and the total solute flux $J_T = J_k + J_u$, so that overall osmotic conservation is

$$\mathbf{F_{v0}\, C_{T0} - J_T = F_{v1}\, C_{T1}}$$

which may be rewritten to provide the ratio of final to initial volume flow

$$\frac{F_{v1}}{F_{v0}} = \frac{C_{T0}}{C_{T1}} - \frac{J_T}{C_{T1} F_{v0}} = \frac{C_{T0}}{C_{T1}} - \frac{C_{k0}\rho_k}{C_{T1}} - \frac{C_{u0}\rho_u}{C_{T1}}$$

in which the fractional reabsorption of either KCl or urea is denoted by $\rho = J / F_{V0}\, C$. The equation for KCl conservation may be used to estimate the initial KCl concentration, as

$$C_{k0} = \frac{F_{v1}}{F_{v0}} C_{k1} + \frac{J_k}{F_{v0}} = \left(\frac{C_{T0}}{C_{T1}} - \frac{C_{k0}\rho_k}{C_{T1}} - \frac{C_{u0}\rho_u}{C_{T1}}\right) C_{k1} + C_{k0}\rho_k$$

so that

$$C_{k0} = \frac{C_{T0}}{C_{T1}} C_{k1} + C_{k0}\rho_k \frac{C_{u1}}{C_{T1}} - C_{u0}\rho_u \frac{C_{k1}}{C_{T1}}$$

In this formula, the first term on the right is the standard TTKG estimate. The second term indicates that

when there is KCl reabsorption, the initial KCl will be underestimated by the TTKG. Conversely, the significance of the third term is that urea reabsorption leads to overestimation of initial KCl by the TTKG. As a consequence, when there is comparable KCl and urea handling by the collecting, the TTKG may retain its accuracy.

Mathematical modeling of in vivo results

In the mouse experiments of this study, we seek to relate changes in urine composition to distal nephron physiology. In this regard, a mathematical model of rat distal nephron may be useful for inferring changes tubular transport during our experimental maneuvers [1]. The model is comprised of epithelial models of distal convoluted tubule (DCT), connecting segment (CNT), and cortical (CCD), outer medullary (OMCD), and inner medullary collecting ducts (IMCD). For the rat kidney, there are 36000 DCT, which coalesce in CNT to 7200 CCD, and then the final reduction in tubule number comes within IMCD. In this model, blood solute concentrations are specified in cortex and medulla, and the model solves for luminal, epithelial, and interstitial concentrations; the solutes of interest are Na+, K+, Cl-, HCO3-, H2CO3, CO2, HPO4-, H2PO4-, urea, NH3, and NH4+. Solute concentrations in tubule fluid entering DCT, and within peritubular blood, are those specified in table 1 of this study [1], with several modifications. In order to represent metabolic alkalosis of the present studies, lumen and blood HCO3 were increased from 8 and 24 mM to 30 and 30 mM; CO2 remained at 1.5 mM. In order to represent the condition of K+ loading, luminal K+ concentration was increased from 2 to 4 mM; peritubular K+ concentration at the outer-inner medullary junction was increased from 10 to 20 mM; and the papillary tip K+ concentration was increased from 20 to 40 mM. The Na+-avid state of the low-Na+ diet was represented by a 2-fold increase in all principal cell transporters within CNT, CCD, and OMCD. Diuretic effects have been represented previously [1]: administration of HCTZ was a 99% reduction in activity of the DCT NaCl cotransporter; amiloride effect was a 99% reduction in ENaC activity of CNT, CCD, and OMCD, and of the non-specific (Na+ and K+) cation channel within the luminal membrane of IMCD.

At baseline, model predictions of urine composition were quite different from the experimental results, and several adjustments to the model parameters were examined. The results of the model simulations are displayed in the table S1, with urine composition in the first 5 data columns, and urine flows of volume and solute in the next 4 columns. Results from simulations using the published parameters, without and with 2-fold increases in principal cell transport, are shown in the top section. At baseline, urine Na+ is too high and urine

K+ is too low; urine osmolality is about half the measured value. When there is increased principal cell activity, there is some improvement in the urine electrolytes, but they are still far from observation. Urine osmolality basically reflects the choice of inner medullary blood tonicity; it will not change in any of these simulations, and for our purposes, it is less critical than the electrolytes. The diuretic simulations are done using the parameter set with (2-fold) higher principal cell activity. Of note, despite the fact that HCTZ produced a nearly doubling in urine flow and urine Na+, there was no significant change in urine Na+ concentration, and this observation was concordant with experimental observation. The implication from this observation is that final urine Na+ concentration depends upon segments distal to DCT, so that changes in DCT transport cannot be inferred from changes in urine composition. As expected, amiloride is a potent diuretic for this region, producing a 4-fold increase in urine Na+ excretion while halving urine K+. Of note, the delivered K+ flow to DCT in this model is 0.86 mol/min, and with amiloride, excreted K+ is about 1/3 less than the delivered load. This reflects CD K+ reabsorption beyond the CNT, which has been a consistent finding in rat micropuncture [2;3].

Strieter *et al*. [4] explored a mathematical model of the CCD in order to identify possible mechanisms for the renal K+ wasting of metabolic alkalosis. Their conclusion was that the best fit to experimental observation came with pH-dependence of both ENaC and ROMK (increased open probability with cytosolic alkalinization), in conjunction with decreased tight junction Cl- permeability with lateral interspace alkalinization. (The tight junction adjustment allowed a shift from NaCl transport to Na+/K+ exchange, with less perturbation of overall Na+ reabsorption.) In the current work, these three parameter features have been incorporated into the principal cell segments of the model (CNT, CCD, and OMCD), and the results appear in the second section of table S1. The pH-dependence of these permeabilities produced substantial reductions in urine Na+ concentration and coordinate increases in urine K+, but still not quite the magnitude that was found in the mouse studies. In order to protect the luminal composition that was established in the cortex, we maintained the pH-dependent parameter set, and then decreased CD tight junction permeabilities by 50% and returned CD principal cell luminal membrane K+ permeabilities (ROMK) back to baseline. The impact of these changes appears in the third section of the table. With this tightening of tight junctions, the urine Na+ reduces to observed levels, and the urine K+ increases further; when the difference in urine osmolality is factored in, urine K+ concentrations are close to the experimental observations. A final adjustment in model parameters took cognizance of the fact the hyperkalemic alkalosis should act to turn off luminal membrane H,K-ATPase. Its

activity in alpha cells (CNT, CCD, and OMCT) and in the IMCD luminal membrane was dropped by 75% (keeping all of the antecedent parameter changes), and those results appear in the bottom section of the table.

With this final adjustment, all of the K+ delivered to DCT appears in the urine when amiloride is applied; the final urine is more alkaline, and thus closer to observation. Figure S2 displays the segmental bookkeeping of Na+, K+, and urea for this distal nephron model, using the final parameter set. For each solute, the left-most bars are distal delivery (DCT entering flow), and the right-most bars display urinary excretion. The 5 sets of interior bars show segmental secretion as positive deflections and reabsorption as negative deflections. The segmental additions and subtractions are summed with delivery to yield excretion. Each set of bars has 4 members, corresponding to baseline parameters, increased principal cell density, and application of the two diuretics. The most important observations are: Na+ reabsorption is nearly complete by the end of CNT; K+ secretion is complete by the end of CNT, with little flux in CCD, and more distal reabsorption; and about half the urea is reabsorbed along medullary CD. The prominent role of CNT in K+ secretion, and the inability of the water permeable CCD to further augment luminal K+ flow have been addressed in prior modeling studies [5;6].

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Supplemental Table S1.

	Final Urine Concentrations (mM)					Excretion Rates (µmol/min)				Isotonic K Conc.*	
Published Model (Weinstein, 2008)										**Estimate**	**Actual**
	Na	K	HCO3	Urea	Osm	Volume	Na	K	Urea	K	K
Baseline	195.6	115.0	77.8	329.4	940	12.1	2.37	1.39	3.99	36.7	40.3
Principal x2	134.2	199.5	53.7	393.1	1021	5.3	0.71	1.05	2.08	58.6	68.8
HCTZ	126.4	177.5	41.5	342.7	955	10.4	1.31	1.84	3.56	55.8	65.7
Amiloride	285.1	25.8	100.0	278.3	888	21.2	6.04	0.55	5.90	8.7	8.1
pH-dependent permeabilities											
	Na	K	HCO3	Urea	Osm	Volume	Na	K	Urea	K	K
Baseline	71.6	248.6	52.6	364.7	990	7.8	0.56	1.95	2.85	75.3	85.9
Principal x2	97.2	249.3	18.3	427.7	1073	2.9	0.28	0.72	1.23	69.7	84.6
HCTZ	53.2	259.4	21.0	384.6	1015	6.0	0.32	1.54	2.29	76.7	93.6
Amiloride	278.8	34.6	96.0	292.4	905	18.2	5.08	0.63	5.33	11.5	14.1
pH-dependent perm / tight CD											
	Na	K	HCO3	Urea	Osm	Volume	Na	K	Urea	K	K
Baseline	71.6	248.6	52.6	364.7	990	7.8	0.56	1.95	2.85	75.3	85.9
Principal x2	19.8	318.0	26.3	399.5	1044	4.7	0.09	1.48	1.87	91.4	85.5
HCTZ	10.4	300.3	21.6	353.3	992	8.9	0.09	2.68	3.16	90.8	93.8
Amiloride	272.2	40.7	106.8	299.1	907	17.0	4.61	0.69	5.07	13.4	14.2
pH-dependent perm / tight CD / H,K-ATPase at 25% baseline											
	Na	K	HCO3	Urea	Osm	Volume	Na	K	Urea	K	K
Baseline	70.1	253.6	65.4	356.8	983	8.6	0.61	2.19	3.09	77.4	87.1
Principal x2	19.7	324.3	43.2	390.7	1034	5.3	0.11	1.73	2.09	94.1	87.2
HCTZ	10.6	306.9	31.5	347.5	986	9.6	0.10	2.95	3.34	93.4	95.9
Amiloride	266.0	48.7	114.2	295.3	905	17.6	4.70	0.86	5.21	16.2	15.1

Supplement Table S1. Urine Concentrations and Flows with Alternative Model Parameters and Experimental Maneuvers

Potassium Concentrations When Tubule Fluid is Isotonic. * Estimated isotonic K concentration is computed according to the standard formula for TTKG (see Supplement 2); the actual isotonic K concentration is obtained by finding the point at which tubule fluid osmolality is 300 mOsm.

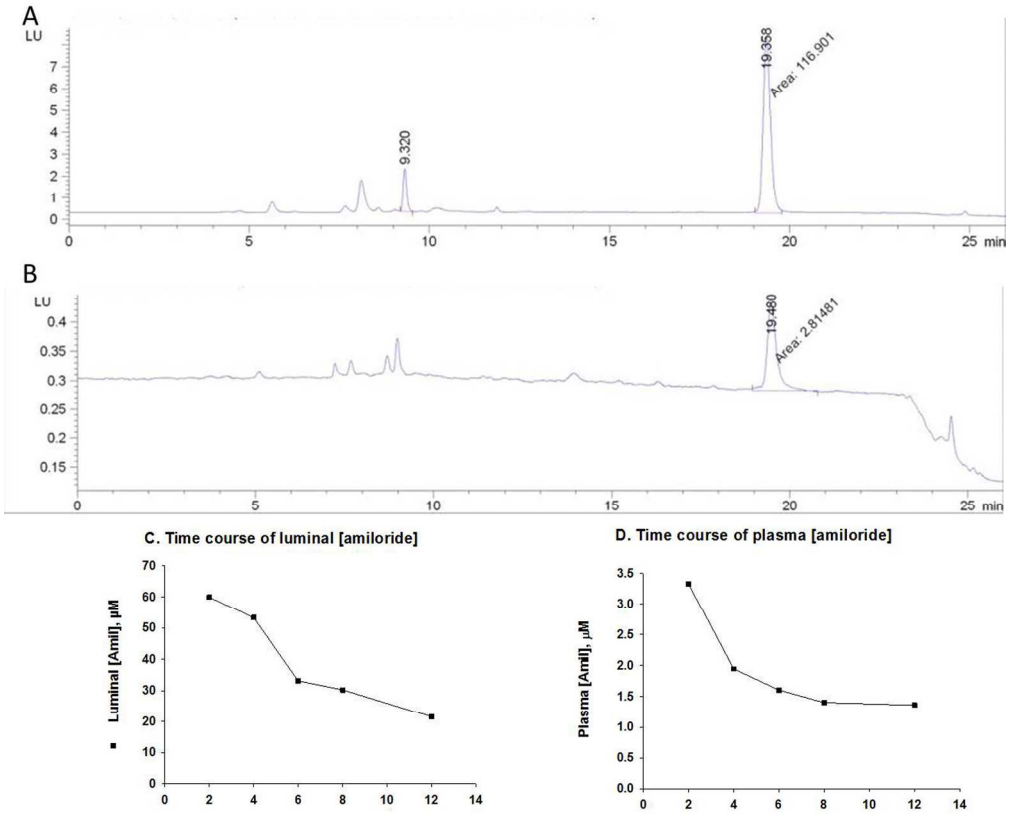

Figure S1. HPLC traces of absorbance at 361 nm vs. time for (A) a urinary sample taken 2 hrs after bolus and (B) a plasma sample taken 6 hrs after bolus, showing elution of amiloride with a defined peak at 16.7 min. (C). Plot of the luminal amiloride concentration vs. time. (D). Plot of plasma amiloride concentration vs. time.
221x182mm (300 x 300 DPI)

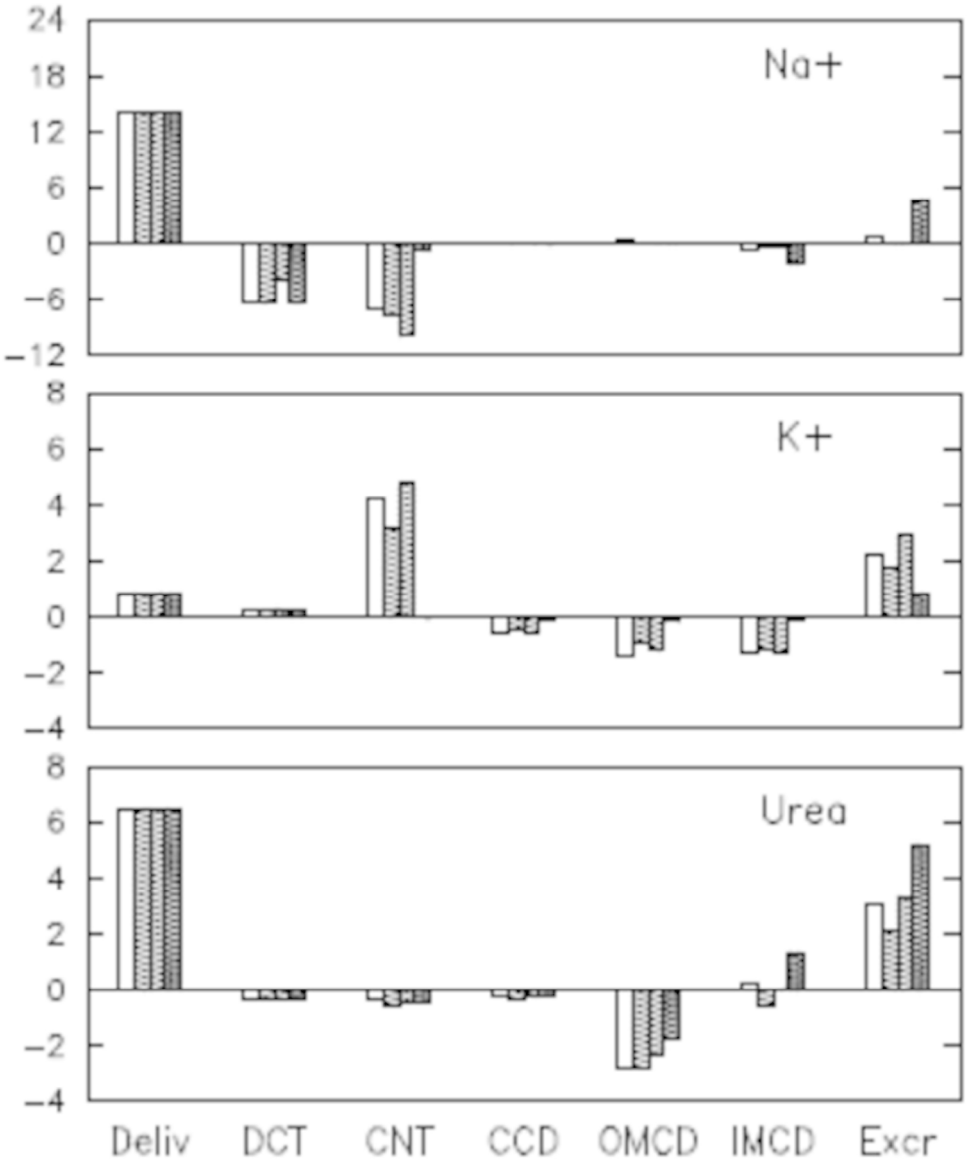

Figure S2. Na, K and urea transport along the distal nephron. Positive bars correspond to delivery or secretion. (μmol/min)
190x224mm (300 x 300 DPI)