Urinary Organic Anion Excretion in Response to Dietary Acid and Base Loading$^{1,2}$

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(J. Am. Soc. Nephrol. 1995; 5:1624-1629)

ABSTRACT

Animals eating a base-loaded or base-forming diet excrete urine containing large amounts of organic anions (OA). Although citrate is the only OA previously identified as being excreted in appreciable amounts during base loading, citrate excretion accounts for only part of total OA excretion. The objectives of this study were to identify other OA excreted by rats and to see how their excretion changed in response to moderate (8 μEq/g per day) and heavy (30 μEq/g per day) loads of NaHCO$_3$ and NH$_4$Cl. Urinary OA were identified by high-performance liquid chromatography and were measured by enzymatic techniques as well. It was found that, in addition to citrate, significant quantities of α-ketoglutarate (α-KG) were excreted by base-loaded rats and that the excretion of citrate, α-KG, and succinate increased with base loading and decreased with acid loading. Citrate plus α-KG excretion rates were, respectively, two-thirds and one-third the rate of HCO$_3^-$ excretion in rats given moderate and heavy base loads. The excretion of creatinine, glutamine, and hippurate showed no clear pattern in response to acid or base loading. It was concluded that, especially in animals experiencing moderate base loads, increases in the excretion of citrate and α-KG represent a much more significant component of base excretion than has been recognized previously.

Key Words: Organic anion excretion, renal acid-base balance, citrate, α-ketoglutarate

In an earlier study (1), it was shown that, in rats, total urinary organic anion excretion varied with the acid and base content of diet. Urinary organic anion excretion paralleled changes in urinary bicarbonate concentration, increasing in response to the addition of NaHCO$_3$ to the diet (base loading) and decreasing in response to the addition of NH$_4$Cl (acid loading). Because organic anions such as citrate and α-ketoglutarate are ordinarily metabolized in the Krebs cycle to yield bicarbonate, in terms of systemic acid-base balance, their excretion can be viewed as being equivalent to bicarbonate ion excretion.

In that study, the total urinary organic anion concentration was measured by a modified version (2) of the titration method described by Van Slyke and Palmer (3). Previous investigations had revealed significant increases in citrate excretion in response to alkalosis (4-7). When the large amounts of total organic anion excreted by the base-loaded animals (1) were compared with previously published rates for citrate excretion in rats given similar base loads (8), it appeared likely that citrate excretion could account for only part of the total organic anion excreted. The excretion of α-ketoglutarate had also been reported to vary with acid-base status. Balaguna and Pitts (9) found that the renal excretion of α-ketoglutarate decreased in dogs in response to respiratory or metabolic acidosis as a result of increased tubular reabsorption. Much more recently, Martin et al. (10) demonstrated that, although in normal rats there was net uptake of α-ketoglutarate by the kidneys, acute alkalosis caused the kidneys to release α-ketoglutarate into both urine and peritubular blood. Neither of those studies addressed the significance of the excretion of α-ketoglutarate in terms of systemic acid-base balance.

Because organic anions other than citrate appeared to be excreted in significant quantities by base-loaded animals, we conducted experiments in which urinary organic anions were identified and quantified in rats fed acid- and base-supplemented diets. To document changes in organic anion excretion over a range of acid and base loads, our animals were exposed to moderate (8 μEq/g per day) and heavy (30 μEq/g per day) acid and base loads.

MATERIALS AND METHODS

Experimental Design

Male Sprague-Dawley rats, weighing between 225 and 265 g, were placed in metabolism cages and allowed to acclimate for at least 24 h, after which, they were assigned to one of five groups. The metabolism cages were maintained at 20 to 22°C and were exposed to a photoperiod of 12 h light–12 h dark, with lights on at 7:00 a.m.

Five groups of six rats each were fed 15 g/day of powdered food (Purina Laboratory Rodent Chow, No. 5001; Indianapolis, IN), which was made into a paste by the addition of 20 mL of water containing 8 μEq/g body wt per day of NaHCO$_3$, NaCl, or NH$_4$Cl (moderate load) or 30 μEq/g body wt per day of NaHCO$_3$ or NH$_4$Cl (heavy load) along with 0.35 g of Equal (NutraSweet brand sweetener; Deerfield, IN) to improve pal-
Analitical Procedures

Blood pH was measured with a Radiometer (Radiometer American, Westlake, OH) BMS2 Mk2 blood microsystem in conjunction with a Radiometer PHM71 MKII acid-base analyzer. Plasma total CO$_2$ was determined enzymatically (Sigma kit #132; Sigma Chemical Co., St. Louis, MD). Urine pH was measured immediately with a gel-filled electrode connected to a Fisher Accumet 620 meter (Fisher Scientific, Pittsburgh, PA). Urine samples were then stored frozen at -80$^\circ$C for later analysis.

Organic anions excreted in appreciable quantities by base-loaded animals were identified by high-performance liquid chromatography (HPLC). The Waters (Millipore Corp., Milford, MA) HPLC system used consisted of a Model 510 dual piston pump, a Bondapak C$_{18}$ reverse phase column, a Model 450 variable wavelength detector (operated at 214 nm), and a Model 745 data module. The mobile phase, pumped at 1 mL/min contained 6% acetonitrile, 0.01 M K$_2$PO$_4$ and 5 mM tetrabutylammonium phosphate, adjusted to pH 7.5. Twenty microliters of filtered (0.22 μ), Whatman (Whatman International, Maidstone, England) Type GS) sample was injected into the chromatography system, and urinary organic anions were tentatively identified by chromatography with known standards. Except for hippurate, the actual concentrations of anions in urine were then determined by a variety of enzymatic analyses. Urinary hippurate concentrations were calculated from HPLC chromatograms (Figure 1) by comparing the ratio of the area under the curve for hippurate with that for the internal standard, acetylsalicylic acid (Sigma).

Urine levels of citrate, α-ketoglutarate, glutamine, and succinate were determined enzymatically by techniques described previously (11). In all four assays, the decrease in the absorbance of NADH at 340 nm was used for quantification. Absorbance was measured with a Milton Roy (Milton Roy Co., Rochester, NY) Model 501 spectrophotometer. All enzymes and reagents were purchased from Sigma Chemical Company, except for succinate thiokinase, used in the succinate assay, which was obtained from Boehringer-Mannheim (Indianapolis, IN). The succinate thiokinase we used is prepared from pig heart and requires GTP rather than ATP as a reactant.

The creatinine concentration of urine was measured by the alkaline picrate method. Urine total CO$_2$ was measured either enzymatically or directly with a CO$_2$ analyzer (Corning Model 965; Corning, NY). Urine bicarbonate ion concentrations were estimated from the total CO$_2$ measurements, correcting for the degree of dissolution and dissociation.

Chloride ion levels of urine were measured by the thiocy-
CO₂ values reported. However, in a different series of experiments in which rats were subjected to the same heavy acid and base loads as in this study and in which blood was also collected from anesthetized rats, base loading had no significant effect on blood pH after 1, 2, or 4 days of loading. On the other hand, rats given heavy acid loads had significantly lower blood pH after 1 and 2 days of loading, but by 4 days of loading, blood pH had returned to control levels (14).

As expected, urinary excretion was substantially changed by treatment. Both levels of acid and base loading caused the expected significant changes in urine pH, HCO₃⁻ excretion, and NH₄⁺ excretion (P < 0.05; Tables 1 through 4).

Organic Anion Excretion

HPLC analysis of urine from controls and from rats given moderate acid and base loads revealed that appreciable quantities of citrate, α-ketoglutarate, creatinine, hippurate, and glutamine were excreted (Table 2). Both HPLC and enzymatic analyses showed that only small quantities (<0.15 μEq/g per day) of lactate, oxalate, pyruvate, and β-hydroxybutyrate were excreted (data not shown).

Along with HCO₃⁻, the excretion of citrate and α-ketoglutarate increased as urine pH increased. Heavy base loading caused an approximately three-fold increase in citrate and α-ketoglutarate excretion from control levels, whereas HCO₃⁻ excretion increased by about an order of magnitude (Table 2). In rats given the heavy acid load, the excretion of HCO₃⁻, citrate, and α-ketoglutarate was nearly zero. Hippurate excretion was unaffected by the treatments, and although there were small significant changes in creatinine and glutamine excretion, there was no clear pattern (Table 2).

Late in this study, we suspected that succinate excretion might vary with acid and base loading, so it was measured in animals subjected to heavy acid and base loads. Succinate excretion was nearly zero in acid-loaded animals. Heavy base loading resulted in the excretion of only 0.37 ± 0.04 μEq/g per day of succinate, a rate much less than that for either citrate or α-ketoglutarate.

In an earlier study, it was shown that the excretion of NH₄⁺, Na⁺, and Cl⁻ varied with diet in animals given the moderate acid and base loads (1). In this investigation, we found also that the excretion of NH₄⁺, Na⁺, and Cl⁻ varied with the heavy dietary loads, but that there were no significant differences in potassium, phosphate, or sulfate ion excretion rates (t test, P < 0.05; Table 3).

DISCUSSION

Excretion of HCO₃⁻ and Trichloroacetic Acid Cycle Intermediates

In response to acid loading, protons are secreted into the proximal tubule fluid. The combination of protons with bicarbonate ions yields CO₂, which is reabsorbed. Thus, bicarbonate is conserved and urinary bicarbonate excretion approaches zero when the kidney is excreting an acid load (Table 2). By contrast, when animals are fed an alkaline diet, HCO₃⁻ excretion rates can be quite high (Table 2).

TABLE 1. Body Weight, Salt Intake, and Acid-Base Values

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Wt (g)</th>
<th>Salt Intake (μEq/g per day)</th>
<th>Urine pH</th>
<th>Blood pH</th>
<th>Plasma Total CO₂ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy Base Load</td>
<td>248 ± 4</td>
<td>NaHCO₃, 30</td>
<td>8.50 ± 0.08b</td>
<td>7.38 ± 0.01</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>Moderate Base Load</td>
<td>241 ± 5</td>
<td>NaHCO₃, 8</td>
<td>7.90 ± 0.09b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Control</td>
<td>240 ± 4</td>
<td>NaCl, 8</td>
<td>6.94 ± 0.03b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Moderate Acid Load</td>
<td>239 ± 3</td>
<td>NH₄Cl, 8</td>
<td>6.23 ± 0.05b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Heavy Acid Load</td>
<td>251 ± 3</td>
<td>NH₄Cl, 30</td>
<td>5.71 ± 0.16b</td>
<td>7.38 ± 0.02</td>
<td>24 ± 2</td>
</tr>
</tbody>
</table>

All values are mean ± SE. ND, not determined.

Mean urine pH values are all significantly different from one another at the 0.05 level (one-way analysis of variance and Duncan's test).

TABLE 2. Urinary Organic Anion Excretion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HCO₃⁻</th>
<th>Citrate</th>
<th>α-KG⁺</th>
<th>Succ</th>
<th>Cr</th>
<th>Gln</th>
<th>Hipp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy Base Load</td>
<td>15.16 ± 0.87</td>
<td>3.68 ± 0.27</td>
<td>1.84 ± 0.15</td>
<td>0.37 ± 0.04</td>
<td>0.49 ± 0.03</td>
<td>0.28 ± 0.01</td>
<td>0.33 ± 0.05</td>
</tr>
<tr>
<td>Moderate Base Load</td>
<td>3.18 ± 0.37</td>
<td>1.29 ± 0.16</td>
<td>0.82 ± 0.04</td>
<td>ND</td>
<td>0.55 ± 0.04</td>
<td>0.87 ± 0.05</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>Control</td>
<td>1.45 ± 0.18</td>
<td>1.11 ± 0.10</td>
<td>0.61 ± 0.05</td>
<td>ND</td>
<td>0.48 ± 0.03</td>
<td>0.71 ± 0.14</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>Moderate Acid Load</td>
<td>0.03 ± 0.02</td>
<td>0.74 ± 0.13</td>
<td>0.60 ± 0.04</td>
<td>ND</td>
<td>0.33 ± 0.02</td>
<td>0.58 ± 0.04</td>
<td>0.38 ± 0.09</td>
</tr>
<tr>
<td>Heavy Acid Load</td>
<td>0.003 ± 0.001</td>
<td>0.10 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.42 ± 0.03</td>
<td>0.34 ± 0.04</td>
<td>0.24 ± 0.02</td>
</tr>
</tbody>
</table>

Abbreviations used are α-KG, α-ketoglutarate; Succ, succinate; Cr, creatinine; Gln, glutamine; Hipp, hippurate; ND, not determined. Units for all values are microequivalents per gram per day (means ± SE). Mean values within each column that are adjacent to the same vertical bar on the left are not significantly different from one another at the 0.05 level (one-way analysis of variance and Duncan's test).
TABLE 3. Urinary Excretion of Ammonia and Electrolytes In Response to Heavy Acid and Base Loadsa

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NH4⁺</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Cl⁻</th>
<th>PO₄</th>
<th>SO₄²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy Base Load</td>
<td>0.27 ± 0.14</td>
<td>26.61 ± 1.31</td>
<td>12.17 ± 0.56</td>
<td>8.03 ± 0.88</td>
<td>2.87 ± 0.21</td>
<td>4.73 ± 0.26</td>
</tr>
<tr>
<td>Heavy Acid Load</td>
<td>19.13 ± 2.66b</td>
<td>10.44 ± 0.84b</td>
<td>15.20 ± 1.16</td>
<td>38.84 ± 2.98b</td>
<td>3.04 ± 0.51</td>
<td>4.55 ± 0.43</td>
</tr>
</tbody>
</table>

*a Units for all values are microequivalents per gram per day (means ± SE).

b Denotes means significantly different (t test; *P < 0.05) from means for Heavy Base Load group.

TABLE 4. Urinary Ion Balancea

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sum of HCO₃⁻ + citrate + α-KG + Succ</th>
<th>Sum of Cr⁺ + Gln + Hipp + PO₄ + SO₄²⁻</th>
<th>NH4⁺</th>
<th>Sum of Na⁺ + K⁺ - Cl⁻</th>
<th>Sum of All Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy Base Load</td>
<td>21.11 ± 1.21a</td>
<td>8.61 ± 0.04</td>
<td>0.27 ± 0.14</td>
<td>31.05 ± 1.45</td>
<td>1.31 ± 0.99</td>
</tr>
<tr>
<td>Moderate Base Load</td>
<td>5.33 ± 0.39</td>
<td>7.60 ± 0.21b</td>
<td>0.67 ± 0.22b</td>
<td>16.05 ± 2.24b</td>
<td>3.78 ± 0.28</td>
</tr>
<tr>
<td>Control</td>
<td>3.38 ± 0.29</td>
<td>7.52 ± 0.08b</td>
<td>1.47 ± 0.44b</td>
<td>12.14 ± 1.93b</td>
<td>2.70 ± 0.29</td>
</tr>
<tr>
<td>Moderate Acid Load</td>
<td>1.49 ± 0.10</td>
<td>5.96 ± 0.07b</td>
<td>3.07 ± 0.58b</td>
<td>5.63 ± 1.93b</td>
<td>1.37 ± 0.10</td>
</tr>
<tr>
<td>Heavy Acid Load</td>
<td>0.18 ± 0.07</td>
<td>8.66 ± 0.09</td>
<td>19.13 ± 2.56</td>
<td>−13.20 ± 1.38</td>
<td>−2.91 ± 0.13</td>
</tr>
</tbody>
</table>

*a Abbreviations used for names of organic anions are Cr, creatinine; Gln, glutamine; Hipp, hippurate; α-KG, α-ketoglutarate; Succ, succinate. Units for all values are microequivalents per gram per day (means ± SE).

b Values for PO₄, SO₄²⁻, NH₄⁺, Na⁺, K⁺, and Cl⁻ for control rats and for rats given moderate acid and base loads are from Brown et al. (1) in which rats of the same age and strain were subjected to the same dietary acid and base loads under the same experimental conditions used in this study.

Many earlier studies of several species have shown that urinary citrate excretion increases during alkalosis (4–8, 15) and that the increased excretion is due to decreased renal reabsorption (16, 17). Organic anions filtered in the glomerulus are reabsorbed primarily in the proximal tubule (18). In vitro studies using perfused tubules and brush border membrane vesicles have shown that the trichloroacetic acid intermediate citrate, α-ketoglutarate, and succinate are transported across the apical membrane of proximal tubule cells via an Na⁺-dependent process (18–21) and that reabsorption is inhibited by a high luminal pH (22).

A further result of increased proton secretion into proximal tubule fluid is the protonation of titratable groups having pKa values in the appropriate range. Trivalent citrate is protonated to the divalent form (pKa values = 3.12, 4.72, and 6.40). The increased citrate reabsorption and the resulting decreased excretion seen in acid-loaded animals have been hypothesized to be because the apical transporter is specific for the divalent form of citrate (19, 21) so that, as tubular fluid pH falls, more of the filtered citrate is reabsorbed.

Experiments using renal cortical brush border membrane vesicles (20, 23) have shown that citrate, α-ketoglutarate, and succinate competitively inhibit the transport of one another, suggesting that they share the same Na⁺-dependent transporter. In our study, heavy base loading significantly increased the excretion of α-ketoglutarate and succinate, as well as citrate (Table 2), even though α-ketoglutarate and succinate, unlike citrate, remain divalent throughout the physiologic pH range. The cause of their increased excretion cannot be explained by a change in charge, as happens for citrate, and must be due to some other factor. It is interesting to note that Jenkins et al. (24) found an increased capacity of brush border membrane vesicles from NH₄Cl-loaded rats to transport citrate. Citrate transport was measured at a medium pH of 8.5 in vesicles from both control and acid-loaded rats, so the difference in transport rates in that system cannot be explained on the basis of changes in the charge of citrate ions.

It is important to recognize that changes in cellular metabolism might also contribute to changes in renal organic anion excretion (25). More recent work has shown, however, that, in isolated perfused rat kidneys, when the perfusate contained citrate and was alkaline, citrate transport and utilization decreased in the absence of detectable changes in total tissue citrate concentration in vitro (26). It is also possible that changes in citrate or α-ketoglutarate metabolism in organs other than the kidney might affect their excretion. In a series of Investigations, Hood and coworkers have shown that, in fasting humans (27) and in rats (28), alkali loading stimulates and acid loading suppresses the production and subsequent excretion of organic anions, especially the ketoacids, β-hydroxybuturate, and acetocetate.

Role of Organic Anion Excretion in Systemic Acid-Base Balance

It is clear from this and many earlier studies that when mammals are challenged with a heavy dietary base load, the most quantitatively significant renal response is a dramatic increase in urinary HCO₃⁻ excretion. However, in these experiments, the urinary excretion of citrate plus α-ketoglutarate equaled about one-third the number of equivalents of HCO₃⁻ excreted (Figure 2). In animals fed moderate base loads,
such as they might incur by eating a nongrain-based herbivorous diet, the combined number of equivalents of citrate and α-ketoglutarate excreted equalled approximately two-thirds the equivalents of HCO₃⁻ excreted. The excretion of such a large fraction of a base load in the form of organic anions, especially citrate, has important physiologic advantages. The excretion of such a large fraction of a base at a lower urine pH, decreasing the likelihood of calcium stone formation. Because citrate ions complex with calcium ions, high urinary levels of citrate also directly inhibit calcium stone formation. Both factors could be quite important in herbivores eating nongrain-based, alkaline ash diets because they are obliged to excrete alkaline urine. In humans, base loading also causes increased urinary citrate (7) and total organic anion excretion (29), but excretion rates in humans are about two orders of magnitude lower than in base-loaded rats.

Renal Ion Balance

In Table 4, the number of equivalents of all ions measured is summed algebraically to determine how nearly that sum approaches the required electroneutrality of urine. As can be seen in the last column of Table 4, the sums of equivalents of all measured ions deviated from electroneutrality by less than 4 μEq/g per day. In four of the five groups, there appears to be missing anion, and in the fifth group—animals given a heavy acid load—there appears to be about 3 μEq/g per day of cation missing. Despite these variations from electroneutrality, we feel confident that we have measured most of the quantitatively significant urinary ions. Although it is likely that small quantities of many other organic anions were excreted by the rats in this study, we have no evidence that any are excreted by base-loaded rats in quantities comparable to citrate or α-ketoglutarate.

ACKNOWLEDGMENTS

We thank Philip Candals and Whitney Brown for technical help and John Burns, Robert Donaldson, and Mark Knepper for critical reviews of the manuscript.

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