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Inhibition of Glutamine Synthetase in the Mouse Kidney

A NOVEL MECHANISM OF ADAPTATION TO METABOLIC ACIDOSIS*

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As part of a study on the regulation of renal ammoniagenesis in the mouse kidney, we investigated the effect of chronic metabolic acidosis on glutamine synthesis by isolated mouse renal proximal tubules. The results obtained reveal that, in tubules from control mice, glutamine synthesis occurred at high rates from glutamate and proline and, to a lesser extent, from ornithine, alanine, and aspartate. A 48 h, metabolic acidosis caused a marked inhibition of glutamine synthesis from near-physiological concentrations of both alanine and proline that were avidly metabolized by the tubules; metabolic acidosis also greatly stimulated glutamine utilization and metabolism. These effects were accompanied by a large increase (i) in alanine, proline, and glutamine gluconeogenesis and (ii) in ammonia accumulation from proline and glutamine. In the renal cortex of acidotic mice, the activity of phosphoenolpyruvate carboxykinase increased 4-fold, but that of glutamate dehydrogenase did not change; in contrast with what is known in the rat renal cortex, metabolic acidosis markedly diminished the glutamine synthetase activity and protein level, but not the glutamine synthetase mRNA level in the mouse renal cortex. These results strongly suggest that, in the mouse kidney, glutamine synthetase is an important regulatory component of the availability of the ammonium ions to be excreted for defending systemic acid-base balance. Furthermore, they show that, in rodents, the regulation of renal glutamine synthetase is species-specific.

Glutamine synthetase, the enzyme that converts glutamate and ammonia into glutamine, is present in the kidney of rat, sheep, guinea pig, rabbit, mouse, and hamster, but absent in the kidney of man, dog, cat, and pig (1–7). Its activity in the kidney of herbivorous species and of the rat is considered to play a key role in limiting the release of the ammonia generated by the renal cells into both the urine and renal vein (4, 6, 8). In agreement with this view, glutamine synthetase is found to be the most active in the kidney of herbivorous species that excrete alkaline urine and to be less active in the kidney of species such as the rat whose urinary pH is approximately neutral (1, 2, 4). Thus, glutamine synthesis not only detoxifies ammonia, a potentially toxic compound to the nervous system (9), but also contributes to the regulation of systemic acid-base balance by reducing the urinary excretion of ammonia in the form of ammonium ions. Contrasting with this important role in acid-base equilibrium control, it is surprising that metabolic acidosis does not alter glutamine synthetase activity in the rat renal cortex (4, 6, 7); indeed, under this circumstance, one would have expected a decreased activity of this enzyme to favor the renal excretion of ammonium ions.

In the rat nephron, glutamine synthetase activity (6) and mRNAs and protein (10) are restricted to the late part of the proximal tubule, *i.e.* the proximal straight tubule. By contrast, in the mouse kidney, in which glutamine synthetase activity is also present in the cortex (6, 11), the mRNAs have been localized to the entire proximal tubule (12). The latter finding demonstrating a different distribution of glutamine synthetase in the proximal tubules of the rat and mouse, together with the fact that adaptation to metabolic acidosis occurs mainly, if not exclusively, in the early segments of rat renal proximal tubules (13–15), led us to consider that glutamine synthesis and its regulation in mouse renal proximal tubules might be intrinsically different from those commonly known in the rat. This is why, in this study, we decided (i) to investigate the capacity of mouse renal proximal tubules to synthesize glutamine and (ii) to test if metabolic acidosis would modify such synthesis. For this, we used miniaturized techniques recently developed in our laboratory to characterize glutamine utilization and metabolism in isolated renal proximal tubules from fed and fasted mice (16).

Our results demonstrate that mouse renal proximal tubules have the capacity to intensely synthesize glutamine from a variety of amino acids. They also show that, unlike in the rat kidney, metabolic acidosis led to a marked decrease in the glutamine synthetase protein and activity, but not in the corresponding mRNAs in the mouse renal cortex; this decrease was accompanied by a dramatic decrease in the capacity of proximal tubules to synthesize glutamine from potentially physiological substrates of the mouse kidney *in vivo*. By contrast, like in the rat kidney, metabolic acidosis stimulated glutamine utilization and ammonia and glucose production by mouse renal proximal tubules.

EXPERIMENTAL PROCEDURES

Reagents—Glutaminase (grade V) and alkaline phosphatase-conjugated goat anti-mouse IgG were from Sigma (L'Isle d'Abeau Chesnes, France). Other enzymes and coenzymes were supplied by Roche Applied Science (Meylan, France). Superscript II™ reverse transcriptase, platinum *Taq* polymerase, and dNTPs were obtained from Invitrogen (Pontoise, France). The mRNA extraction kit was purchased from Dynal (Oslo, Norway), and primers were obtained from Genset S.A. (Paris, France). L-[U-^{14}C]Glutamic acid was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). The monoclonal antibody to glutamine synthetase was from Transduction Laboratories (Lexington, KY).

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TABLE I

Glutamine synthesis from various L-amino acids (5 mM) in mouse renal proximal tubules

Tubules (0.62 ± 0.15 mg of protein/flask) were incubated for 60 min as described under "Experimental Procedures." Results for metabolite removal (−) or production are reported as means ± S.E. for four experiments performed in duplicate. Statistical difference was measured by Student's *t* test against the control without any added substrate. The nitrogen found was the sum of the glutamate and ammonia plus twice the glutamine found in the presence of each amino acid minus the nitrogen found in the absence of amino acid.

Amino acid	Metabolite production or removal (−)				
	Glutamine	Glutamate	Ammonia	Glucose	Nitrogen found
	nmol/mg protein/h				
None	−5 ± 4	−2 ± 3	152 ± 14	15 ± 2	—
Glutamate	471 ± 24[a]	—	342 ± 79	166 ± 6[a]	1149 ± 125
Proline	305 ± 25[a]	338 ± 48[a]	314 ± 58[a]	208 ± 10[a]	1124 ± 50
Ornithine	84 ± 7[a]	94 ± 9[a]	419 ± 34[a]	38 ± 7[a]	542 ± 25
Alanine	78 ± 8[a]	32 ± 1[a]	679 ± 55[a]	71 ± 9[a]	729 ± 64
Aspartate	22 ± 3[a]	158 ± 11[a]	727 ± 45[a]	67 ± 6[a]	791 ± 60
Isoleucine	10 ± 1[a]	19 ± 2[a]	219 ± 48	16 ± 5	118 ± 40
Valine	6 ± 2[a]	17 ± 4[a]	244 ± 56[a]	18 ± 5	134 ± 50
Leucine	5 ± 2[a]	21 ± 6[a]	399 ± 74[a]	27 ± 8	292 ± 61
Arginine	4 ± 6	1 ± 4	276 ± 19[a]	28 ± 1[a]	145 ± 27
Methionine	3 ± 3	17 ± 5[a]	299 ± 63[a]	9 ± 2[a]	183 ± 51
Cysteine	−3 ± 5	1 ± 4	224 ± 53	2 ± 2[a]	80 ± 46
Asparagine	−7 ± 6	6 ± 2[a]	428 ± 22[a]	11 ± 1[a]	282 ± 15
Histidine	−7 ± 6	−5 ± 5	192 ± 30	6 ± 2[a]	35 ± 25

[a] $p < 0.05$.

Mice—Female Swiss mice (OF1; 28–32 g) were obtained from Institut Français de la Fièvre Aphteuse-Centre de Recherche et d'Elevage des Oncins (Saint-Germain-sur-l'Arbresle, France). The animals were fed a standard diet (Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge, France) and either had free access to water or were given 0.28 mol/liter NH_4Cl in the drinking water for 48 h. This treatment led to metabolic acidosis as demonstrated by a 3.6-fold increase in total urinary acid excretion (mainly in the form of ammonium ions) determined as described previously (16).

Preparation of Renal Proximal Tubules and Incubations—Renal proximal tubules were prepared by collagenase treatment of renal cortex slices as described by Conjard *et al.* (16). Incubations were performed for 30 and 60 min at 37 °C in a shaking water bath in 12-ml stoppered Erlenmeyer flasks in an atmosphere of O_2/CO_2 (19:1). Tubules were incubated in 1 ml of Krebs-Henseleit medium with or without various L-amino acids as substrate. The flasks were prepared in duplicate or triplicate for all experimental conditions. Incubation was stopped by adding perchloric acid (3% (v/v) final concentration) to each flask. In all experiments, zero-time flasks with and without substrate were prepared by adding perchloric acid before the tubules. After removal of the denatured protein by centrifugation, the supernatant was neutralized with 20% (w/v) KOH for metabolite determination.

Metabolite Assays—Glucose, lactate, pyruvate, glutamate, glutamine, alanine, aspartate, citrate, α-ketoglutarate, fumarate, malate, ammonia, and ATP were determined by the methods described by Passonneau and Lowry (17). The protein content of the tubules added to each flask was determined by the bicinchoninic acid protein assay (18).

Measurement of Enzyme Activities—To measure glutamine synthetase activity, a piece of renal cortex was homogenized at 4 °C at a final concentration of 25 mg/ml tissue fresh weight in an appropriate buffer containing 10 mM imidazole-HCl (pH 7.0) and 0.5 mM EDTA. Homogenates were centrifuged at 10,000 × *g* for 10 min, and 30 μl of a properly diluted aliquot of the supernatant was used for activity measurement. Glutamine synthetase activity was measured over a 30-min incubation period at 37 °C according to a radioactive method adapted from that of Prusiner and Milner (19). Briefly, the assay contained (final concentrations) 75 mM imidazole HCl (pH 7), 10 mM glutamate, 4 mM NH_4Cl, 13 mM $MgCl_2$, 15 mM ATP, 1 mM β-mercaptoethanol, 6 mM phosphocreatine, 13 μg/ml creatine kinase, and 800 Bq of L-[U-^{14}C]glutamic acid (10.5 GBq/mmol) per reaction. Reactions were stopped by heating at 97 °C for 8 min. L-[U-^{14}C]Glutamine was isolated for counting by passing the reaction mixture over an acetate resin (AG® 1-X8, 200–400 mesh, Bio-Rad). The eluate was transferred into a counting vial, and 3 ml of UltimaGold® (PerkinElmer Life Sciences) was added for radioactivity measurement in a Tri-Carb® 2100 TR liquid scintillation analyzer (PerkinElmer Life Sciences). The radioactive [U-^{14}C]glutamic acid used was purified by ion exchange chromatography (acetate resin) to get rid of unspecific ^{14}C-labeled products that may cause some blank. The linearity of activity with time was checked by incubating samples for 15 and 30 min. The relationship of enzyme activity to protein amount (15 and 30 μg) was also linear. Reaction blanks (at zero time or after incubation without ATP) were negligible.

Semiquantitative Analysis of Glutamine Synthetase mRNA Expression—To determine the glutamine synthetase and β-actin mRNA levels in the renal cortex from control and acidotic mice, mRNA was extracted, and semiquantitative reverse transcription-PCR was performed as described (16). A primer pair was selected from the published cDNA sequence of mouse glutamine synthetase (GenBank™/EBI accession number NM_008131). The primers were as follows: forward, 5′-AATGGACATGGTGAGCAACC-3′; and reverse, 5′-GTATGGCAGCCTGCAACATT-3′. The number of PCR cycles for glutamine synthetase was 21. The specific primers for glutamine synthetase were included in the PCR mixture at the start of the amplification. After three cycles of amplification, the specific primers for β-actin (16) were added, and β-actin cDNA was co-amplified with the glutamine synthetase cDNA for the 18 remaining cycles. The PCR product obtained with the primers specific for glutamine synthetase was sequenced by Genome Express (Grenoble, France) and found to be identical to the published mouse glutamine synthetase sequence. Glutamine synthetase mRNA levels are reported relative to β-actin.

Measurement of Glutamine Synthetase Protein Levels in the Mouse Renal Cortex—The glutamine synthetase protein levels in the renal cortex of normal and acidotic mice were measured by SDS-PAGE and immunoblotting. Renal cortical pieces were homogenized in 25 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$ and 300 mM saccharose. Aliquots were heated for 3 min at 95 °C in SDS sample buffer and subjected to SDS gel electrophoresis (25 μg of protein/lane, 12% acrylamide) (20). Western blotting was performed essentially as described by Kyhse-Andersen (21). Proteins were transferred onto polyvinylidene difluoride membranes using a semidry transfer apparatus (Schleicher & Schüll) according to the manufacturer's instructions and blocked for 60 min in 5% dried skim milk in Tris-buffered saline (20 mM Tris-HCl (pH 7.5) and 150 mM NaCl). Blots were incubated at room temperature with the primary antibody against glutamine synthetase (1:1000 dilution) for 60 min, followed by alkaline phosphatase-conjugated secondary antibody (1:10,000 dilution) for 60 min, and developed with ECF™ substrate (Amersham Biosciences) for 10 min. Bands were visualized with a FluorImager™ and quantified with ImageQuant™ software.

Calculations—Net substrate utilization and product formation were calculated as the difference between the total flask contents (tissue plus medium) at the start (zero-time flasks) and after the period of incubation. The metabolic rates, reported as means ± S.E., are expressed in nanomoles of substance removed or produced per mg of tubular protein per unit time (60 min). The results were analyzed by Student's *t* test for unpaired data, comparing values obtained in control mice with those in 48-h acidotic mice, and only results of $p < 0.05$ were considered as statistically significant.

RESULTS

Glutamine Synthesis from Various Amino Acids

Table I shows the results of experiments performed to identify, among the physiological amino acids, the potential precursors of glutamine in renal proximal tubules isolated from fed

TABLE II

Effect of metabolic acidosis on the metabolism of 0.5 and 1 mM alanine in mouse renal proximal tubules

Renal proximal tubules from control (0.42 ± 0.04 and 0.48 ± 0.03 mg of protein/flask with 0.5 and 1 mM alanine, respectively) and acidotic (0.49 ± 0.05 and 0.50 ± 0.06 mg of protein/flask with 0.5 and 1 mM alanine, respectively) mice were incubated for 60 min as described under "Experimental Procedures." Results for metabolite removal (−) or production are reported as means ± S.E. for the number (n) of experiments shown. Statistical difference was measured by unpaired Student's t test, comparing the values found with tubules from acidotic mice with those found with tubules from control mice. The nitrogen found was calculated as the glutamate and ammonia plus twice the glutamine accumulated.

Acid-base status	Metabolite removal (−) or production					
	Alanine	Glutamine	Glutamate	Ammonia	Glucose	Nitrogen found
	nmol/mg protein/h					
0.5 mM alanine						
Control (n = 7)	−295 ± 34	38 ± 5	8 ± 3	371 ± 35	46 ± 8	455 ± 43
Acidotic (n = 6)	−297 ± 25	8 ± 4[a]	7 ± 3	407 ± 37	70 ± 3[a]	428 ± 33
None						
Control (n = 7)	—	1 ± 4	−9 ± 4	137 ± 15	15 ± 2	130 ± 21
Acidotic (n = 6)	—	−3 ± 2	−5 ± 3	188 ± 19	26 ± 3[a]	176 ± 16
1 mM alanine						
Control (n = 5)	−344 ± 44	55 ± 13	8 ± 4	350 ± 41	44 ± 5	469 ± 60
Acidotic (n = 5)	−455 ± 37	12 ± 6[a]	13 ± 4	538 ± 39[a]	99 ± 5[a]	575 ± 33
None						
Control (n = 5)	—	−2 ± 2	−13 ± 5	137 ± 18	13 ± 1	120 ± 21
Acidotic (n = 5)	—	−4 ± 3	−6 ± 4	199 ± 21	28 ± 3[a]	186 ± 25

[a] $p < 0.05$.

mice. For this, a high nonphysiological concentration (5 mM) of these amino acids was used. In these experiments, tubule viability was indicated by the linearity with time of glutamine and glucose synthesis, two ATP-dependent processes, and by the tubular ATP content (16.1 ± 0.7 μmol/g of protein, n = 4).

The most potent glutamine precursor was glutamate, followed by proline, ornithine, alanine, and aspartate. The other amino acids tested were not converted into glutamine or only at very low rates. Large amounts of glutamate accumulated from proline and, to a lesser extent, from aspartate and ornithine. In the presence of all amino acids, ammonia was produced in amounts substantially greater than those found in the absence of any exogenous amino acid, especially in the presence of aspartate, alanine, asparagine, ornithine, and arginine. Glucose was produced at high rates from proline and glutamate and, to a lesser extent, from alanine, aspartate, ornithine, and arginine. In view of the fact that, among the above glutamine precursors, alanine and proline are present in the circulating blood of the mouse at the highest concentrations, *i.e.* 0.2–0.6 mM (22, 23), we chose these two amino acids as substrates for the next steps of our study.

Alanine Metabolism in Proximal Tubules from Control and Acidotic Mice

With both 0.5 and 1 mM alanine as substrate, alanine utilization was linear with time for up to 60 min of incubation; alanine utilization was accompanied by glutamine, glucose, and ammonia formation, which was also linear with time over a 60-min incubation period (n = at least 2 for each alanine concentration with tubules from both control and acidotic mice) (data not shown). No substantial amounts of pyruvate, lactate, aspartate, and tricarboxylic acid cycle intermediates were found to accumulate in tubules from normal or acidotic mice.

Effect of Metabolic Acidosis—Table II shows the results obtained after 60 min of incubation. Metabolic acidosis did not alter the high rate of alanine utilization observed in tubules from control mice with either 0.5 or 1 mM alanine as substrate. By contrast, at both alanine concentrations, the high rate of glutamine synthesis observed in tubules from control mice was dramatically diminished compared with that in tubules from acidotic mice; this effect was accompanied by a statistically significant stimulation of ammonia accumulation only with 1 (but not 0.5) mM alanine as substrate. Acidosis stimulated glucose synthesis especially with 1 mM alanine as substrate. None of the other metabolite tested was produced in substantial amounts from alanine in tubules from either control or acidotic mice. In the absence of alanine, large amounts of ammonia and small amounts of glucose were synthesized from endogenous substrate, and acidosis led to a stimulation of glucose, but not of ammonia production (Table II).

The nitrogen balance calculations given in Table II show that, with 0.5 and 1 mM alanine as substrate and in tubules from both control and acidotic mice, the nitrogen found as glutamine, glutamate, and ammonia (two nitrogen atoms/glutamine molecule and one nitrogen atom/glutamate and ammonia molecules) exceeded by far the amount of nitrogen taken up as alanine (one nitrogen atom/alanine molecule). This clearly indicates that the metabolism of nitrogen observed in the absence of alanine (mainly in the form of ammonia accumulation) still completely occurred in the presence of alanine. As a matter of fact, the alanine uptake was generally in good agreement with the nitrogen found in the presence of alanine minus that found in its absence (Table II). Therefore, to determine the metabolic fate of alanine, the nitrogenous and carbon products found in the absence of alanine were substracted from those found in its presence.

Fate of Alanine Carbon and Nitrogen—Given that two alanine molecules are needed to form the carbon skeleton of each glutamate, glutamine, and glucose molecule, it appears that complete oxidation of alanine represented the major fate of alanine carbon under all of the conditions studied (see the carbon balance calculations in Table III); glucose and, except in tubules from acidotic mice, glutamine represented the two other major products of alanine metabolism. Under all experimental conditions, ammonia represented the main nitrogenous product of alanine metabolism, whereas glutamine was a substantial nitrogenous product only in tubules from control mice (Table III).

Proline Metabolism in Proximal Tubules from Control and Acidotic Mice

As for alanine as substrate, the accumulation of glutamine, ammonia, and glucose in tubules from control and acidotic mice was linear with time for a 60-min incubation period with both 0.5 and 1 mM proline (data not shown). Again, no substantial amounts of pyruvate, lactate, and tricarboxylic acid cycle intermediates were found to accumulate in tubules from normal or acidotic mice. Negligible amounts of aspartate (1.2 nmol/mg of protein/h, mean value for five experiments) accumulated

TABLE III

Effect of metabolic acidosis on the metabolic fate and pathways of alanine in mouse renal proximal tubules

The values, which were derived from those listed in Table II, are means ± S.E. for the number (n) of experiments shown. The percentage of alanine carbon skeleton (C) or nitrogen (N) removal is given. The carbon balance (complete oxidation of the alanine carbon skeleton) was calculated as the alanine removed minus twice the sum of glutamine, glutamate, and glucose found. The flux through glutamate dehydrogenase was calculated as the sum of the glutamine and ammonia found. Statistical difference was measured by unpaired Student's t test, comparing the values found with tubules from acidotic mice with those obtained with tubules from control mice.

Acid-base status	Alanine removed	Distribution of alanine carbon and nitrogen to:					Flux through glutamate dehydrogenase
		Glutamine	Glutamate	Ammonia	Glucose	Complete oxidation	
		nmol/mg protein/h					
0.5 mM alanine							
Control (n = 7)	295 ± 34	37 ± 5 (C, 25%; N, 25%)	17 ± 2 (C, 12%; N, 6%)	234 ± 29 (N, 79%)	32 ± 7 (C, 22%)	124 ± 25 (C, 42%)	271 ± 29
Acidotic (n = 6)	297 ± 25	11 ± 5[a] (C, 7%; N, 7%)	12 ± 5 (C, 8%; N, 4%)	219 ± 40 (N, 74%)	45 ± 5 (C, 30%)	163 ± 28 (C, 55%)	230 ± 35
1 mM alanine							
Control (n = 5)	344 ± 44	57 ± 12 (C, 33%; N, 33%)	21 ± 2 (C, 12%; N, 6%)	214 ± 25 (N, 62%)	31 ± 4 (C, 18%)	125 ± 19 (C, 36%)	271 ± 31
Acidotic (n = 5)	455 ± 37	15 ± 8[a] (C, 7%, N, 7%)	19 ± 7 (C, 8%; N, 4%)	339 ± 51 (N, 75%)	72 ± 7[a] (C, 32%)	243 ± 40[a] (C, 53%)	355 ± 48

[a] $p < 0.05$.

from 1 mM (but not 0.5 mM) proline in renal proximal tubules from control (but not acidotic) mice.

Effect of Metabolic Acidosis—Table IV shows the results obtained in experiments in which tubules from both control and acidotic mice were incubated with 0.5 or 1 mM proline as substrate. As already seen with alanine as substrate, metabolic acidosis markedly reduced the high rates of glutamine synthesis observed with both 0.5 and 1 mM proline. This effect was associated with a stimulation of ammonia accumulation and glucose production at both concentrations of this amino acid. Again, acidosis stimulated the production of glucose (but not ammonia) in the absence of exogenous substrate (Table IV). The total nitrogen found as glutamine, glutamate, and ammonia was not affected by metabolic acidosis with either 0.5 or 1 mM proline, strongly suggesting that proline utilization, like alanine utilization, is not modified by metabolic acidosis.

Metabolic Fate of Proline Carbon and Nitrogen—Assuming that, as with alanine as substrate, the metabolism of endogenous substrates still fully occurred in the presence of proline as substrate, the removal of proline, for which there is no enzymatic assay available, was calculated as the difference between the nitrogen found in the presence of proline minus that found in its absence (Tables IV and V). Therefore, the accumulation of glutamate and ammonia and the production of glutamine and glucose from proline shown in Table V were calculated as the differences between the values (shown in Table IV) found in the presence and absence of proline.

It can be seen that acidosis did not alter proline utilization (Table V). Glutamine was the main carbon product of proline metabolism in tubules from control (but not acidotic) mice, whereas glucose was the predominant carbon product of proline metabolism in tubules from acidotic mice with both 0.5 and 1 mM proline. Carbon balance calculations revealed that complete oxidation of proline never exceeded 24% of substrate utilization and was not statistically changed by acidosis.

Effect of Ammonium Chloride Acidosis on Food and Water Consumption

The stimulation of glucose synthesis from alanine and proline observed in this study suggested that ammonium chloride acidosis might have reduced the food and water intake in our mice and that fasting might have been responsible, at least in part, for the inhibition of glutamine synthesis also observed in tubules from acidotic mice (Tables II and IV). To address this issue, seven mice were placed in a homemade metabolic cage. They had free access to food for 96 h; after 48 h, the drinking water to which they had also free access was replaced with the ammonium chloride solution (0.28 mol/liter) for the next 48 h. During the control period (0–48 h), the mean food and water consumption per mouse was 8.7 g and 8.7 ml, respectively, and their blood glucose level at 48 h was 7.6 ± 0.1 mM. During the metabolic acidosis period (48–96 h), the corresponding values were 6.9 g and 6.1 ml, and blood glucose level at 96 h was 7.5 ± 0.2 mM.

To verify whether such metabolic acidosis-induced decreases in food and water consumption would alter alanine and proline metabolism, tubules were prepared from five control mice given 8.7 g of food and 8.7 ml of water per mouse over 48 h and from five mice whose food and water consumption was limited over 48 h to 6.9 g and 6.1 ml per mouse. The tubules were incubated with 1 mM alanine or proline as substrate. Food and water restriction did not statistically alter alanine utilization and metabolism by the tubules. In tubules from control mice, alanine utilization; glutamate accumulation; and glutamine, ammonia, and glucose production were 378 ± 38, 33 ± 11, 64 ± 14, 379 ± 42, and 47 ± 5 nmol/mg of protein/h, respectively. In tubules from food- and water-restricted mice, the corresponding values were 343 ± 25, 30 ± 9, 46 ± 5, 338 ± 20, and 35 ± 6 nmol/mg of protein/h. Similarly, neither glutamate accumulation nor glutamine, ammonia, or glucose production was significantly modified by food and water restriction when the tubules were incubated with 1 mM proline. In tubules from control mice, the respective values were 120 ± 19, 196 ± 16, 255 ± 25, and 111 ± 7 nmol/mg of protein/h; in tubules from food- and water-restricted mice, the corresponding values were 107 ± 9, 174 ± 8, 219 ± 39, and 88 ± 10 nmol/mg of protein/h. Thus, the decrease in glutamine synthesis and the stimulation of glucose production from both alanine and proline observed in tubules from acidotic mice were due to metabolic acidosis *per se* and not to metabolic acidosis-induced fasting.

Glutamine Metabolism in Proximal Tubules from Control and Acidotic Mice

Table VI shows that a 48-h metabolic acidosis also markedly increased the high rate of glutamine utilization observed in tubules from control mice. This effect, which indicates a stimulation of flux through glutaminase, the enzyme initiating glutamine degradation, was associated with a large stimulation of ammonia and glucose production, but not of glutamate accumulation. In agreement with the observation that the increase in ammonia production caused by metabolic acidosis was greater than the increase in flux through glutaminase, the

TABLE IV

Effect of metabolic acidosis on the metabolism of 0.5 and 1 mM proline in mouse renal proximal tubules

Renal proximal tubules from control (0.40 ± 0.04 and 0.44 ± 0.03 mg of protein/flask with 0.5 and 1 mM proline, respectively) and acidotic (0.53 ± 0.04 and 0.55 ± 0.04 mg of protein/flask with 0.5 and 1 mM proline, respectively) mice were incubated for 60 min as described under "Experimental Procedures." Results for metabolite removal (−) or production are reported as means ± S.E. for the number (*n*) of experiments shown. Statistical difference was measured by unpaired Student's *t* test, comparing the values found with tubules from acidotic mice with those found with tubules from control mice. The nitrogen found was calculated as the glutamate and ammonia plus twice the glutamine accumulated.

Acid-base status	Metabolite removal (−) or production				
	Glutamine	Glutamate	Ammonia	Glucose	Nitrogen found
	nmol/mg protein/h				
0.5 mM proline					
Control (n = 8)	131 ± 7	43 ± 6	237 ± 32	78 ± 8	542 ± 30
Acidotic (n = 6)	17 ± 6[a]	23 ± 5[a]	430 ± 44[a]	122 ± 8[a]	487 ± 51
None					
Control (n = 8)	4 ± 3	−2 ± 2	143 ± 16	13 ± 3	150 ± 16
Acidotic (n = 6)	−1 ± 2	−7 ± 3	172 ± 9	24 ± 1[a]	164 ± 12
1 mM proline					
Control (n = 6)	221 ± 10	81 ± 13	211 ± 25	98 ± 3	734 ± 29
Acidotic (n = 5)	42 ± 10[a]	55 ± 7	555 ± 41[a]	186 ± 17[a]	695 ± 50
None					
Control (n = 6)	5 ± 3	−4 ± 2	146 ± 20	15 ± 3	152 ± 17
Acidotic (n = 5)	0 ± 2	−8 ± 3	176 ± 9	23 ± 1[a]	168 ± 12

[a] $p < 0.05$.

TABLE V

Effect of metabolic acidosis on the metabolic fate and pathways of proline in mouse renal proximal tubules

The values, which were derived from those listed in Table IV, are means ± S.E. for the number (*n*) of experiments shown. The percentage of proline carbon skeleton (C) or nitrogen (N) removal taken as the nitrogen found in the presence of proline minus that found in its absence is given. The carbon balance (complete oxidation of the proline carbon skeleton) was calculated as the difference between the calculated proline removal and the sum of glutamine, glutamate, and twice the glucose found. The flux of the proline carbon skeleton through glutamate dehydrogenase was calculated as the sum of the glutamine and ammonia found. Statistical difference was measured by unpaired Student's *t* test, comparing the values found with tubules from acidotic mice with those found with tubules from control mice.

Acid-base status	Proline removed	Distribution of proline carbon and nitrogen to:					Flux through glutamate dehydrogenase
		Glutamine	Glutamate	Ammonia	Glucose	Complete oxidation	
	nmol/mg protein/h						
0.5 mM proline							
Control (n = 8)	392 ± 20	127 ± 7 (C, 32%; N, 65%)	45 ± 4 (C, 11%; N, 11%)	94 ± 23 (N, 24%)	65 ± 9 (C, 33%)	91 ± 10 (C, 23%)	221 ± 21
Acidotic (n = 6)	323 ± 41	18 ± 5[a] (C, 6%; N, 11%)	30 ± 6[a] (C and N, 9%)	258 ± 36[a] (N, 80%)	98 ± 9[a] (C, 61%)	79 ± 21 (C, 24%)	276 ± 37
1 mM proline							
Control (n = 6)	582 ± 14	216 ± 11 (C, 37%; N, 74%)	85 ± 11 (C and N, 15%)	66 ± 17 (N, 11%)	83 ± 5 (C, 29%)	115 ± 13 (C, 20%)	281 ± 18
Acidotic (n = 5)	526 ± 44	42 ± 10[a] (C, 8%; N, 16%)	63 ± 10 (C and N, 12%)	379 ± 34[a] (N, 72%)	162 ± 17[a] (C, 62%)	96 ± 40 (C, 18%)	421 ± 36[a]

[a] $p < 0.05$.

TABLE VI

Effect of metabolic acidosis on the metabolism of 0.75 mM glutamine in mouse renal proximal tubules

Renal proximal tubules from control and acidotic mice (0.73 ± 0.02 and 0.83 ± 0.07 mg of protein/flask, respectively) were incubated for 60 min as described under "Experimental Procedures." Flux through glutamate dehydrogenase was calculated as the difference between the glutamine removed and the glutamate accumulated. Results for metabolite removal (−) or production are reported as means ± S.E. for the number (*n*) of experiments shown. Statistical difference was measured by unpaired Student's *t* test, comparing the values found with tubules from acidotic mice with those found with tubules from control mice.

Acid-base status	Metabolite removal (−) or production				
	Glutamine	Glutamate	Ammonia	Glucose	Flux through glutamate dehydrogenase
	nmol/mg protein/h				
0.75 mM glutamine					
Control (n = 5)	−425 ± 29	42 ± 3	805 ± 19	109 ± 6	383 ± 27
Acidotic (n = 5)	−568 ± 30[a]	44 ± 5	1137 ± 38[a]	187 ± 9[a]	524 ± 25[a]

[a] $p < 0.05$.

flux through glutamate dehydrogenase was also found to be stimulated by acidosis (Table VI). The nitrogen balance calculations, taken as the difference between twice the glutamine utilization minus the sum of the ammonia and glutamate found, suggest that most of the metabolism of endogenous substrates observed in the absence of exogenous substrates (Tables II and IV) was inhibited by glutamine. Complete oxidation of the glutamine carbon skeleton, calculated as the difference between glutamine utilization and the sum of glutamate and twice the glucose found, remained unaffected by metabolic acidosis (Table VI).

Glutamine Synthetase Activity and Protein and mRNA Levels

The activity of glutamine synthetase measured in the renal cortex of five acidotic mice was greatly diminished compared with that of four control mice (204 ± 31 *versus* 805 ± 57

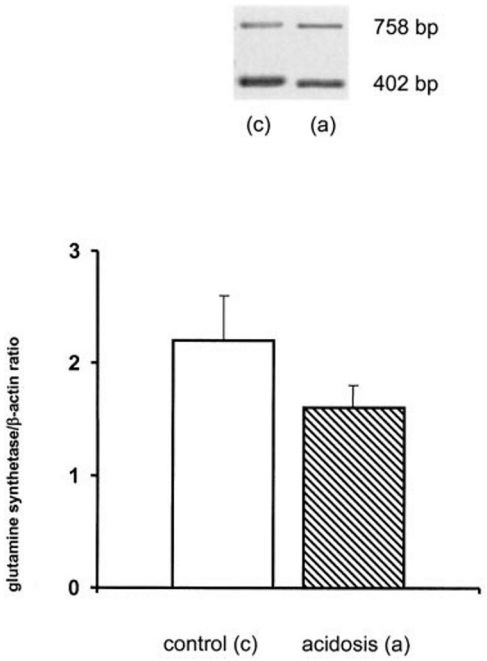


FIG. 1. **Renal cortical mRNA levels of glutamine synthetase and β-actin in control and acidotic mice.** The mRNA levels of glutamine synthetase and β-actin were analyzed by semiquantitative reverse transcription-PCR analysis as described under "Experimental Procedures." The amplified cDNAs (glutamine synthetase, 402 bp; and β-actin, 758 bp) were separated by agarose gel electrophoresis as shown in the *upper panel*. Band intensities were quantified and are reported relative to the β-actin band in the *lower panel*. The glutamine synthetase/β-actin band intensity ratios were 2.2 ± 0.4 and 1.6 ± 0.2 (means ± S.E.) in the renal cortex of control (*c*; $n = 4$) and acidotic (*a*; $n = 5$) mice, respectively. These ratios were not statistically different.

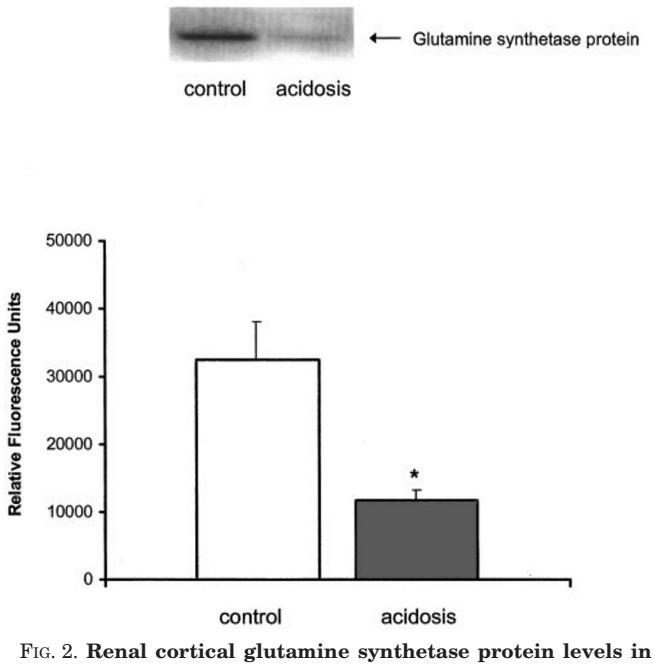


FIG. 2. **Renal cortical glutamine synthetase protein levels in control and acidotic mice.** Renal cortical extracts from control and acidotic mice were subjected to immunoblotting as described under "Experimental Procedures." The immunoblot (*upper panel*) was probed with a monoclonal antibody against glutamine synthetase. The glutamine synthetase expression level (relative fluorescence units) was quantified in the renal cortex of four control and four acidotic mice (*lower panel*). Data are reported as means ± S.E. for four experiments. *, statistically different from the control ($p < 0.05$).

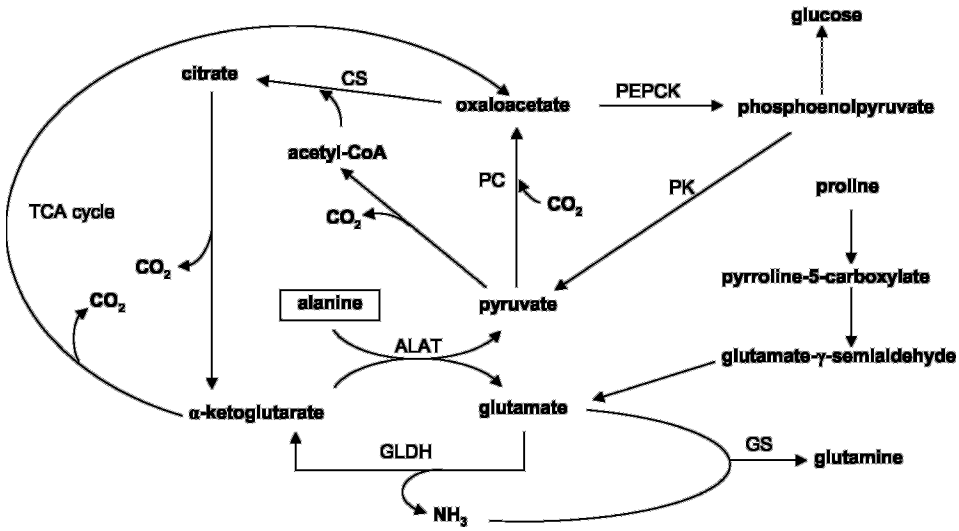
nmol/mg of protein/h, respectively; $p < 0.05$). Fig. 1 shows that the decrease in glutamine synthetase activity was not due to a reduced glutamine synthetase mRNA level. By contrast, Fig. 2 shows that the mean glutamine synthetase protein level was reduced by 64% in the renal cortex of acidotic mice.

DISCUSSION

Glutamine Synthesis from Various Amino Acids—This study demonstrates that, like isolated rabbit and guinea pig renal proximal tubules and renal cortical slices (1, 24–30), isolated mouse renal proximal tubules have the capacity to synthesize substantial amounts of glutamine from 5 mM glutamate, proline, ornithine, alanine, and aspartate. This indicates that, in these tubules, all of the above glutamine precursors are metabolized to a substantial extent by the appropriate enzymes to form the glutamate and ammonia needed for glutamine synthesis. Except for arginine, whose molecule contains four nitrogen atoms, and for asparagine and ornithine, whose molecule contains two nitrogen atoms, calculations of the nitrogen found in the form of glutamine, glutamate, and ammonia corrected for the values found in the absence of the amino acid give an estimate of the minimum amino acid utilization (Table I). It can be seen that glutamate followed by proline, aspartate, and alanine were utilized at the highest rates. Interestingly, large amounts of both glutamate and ammonia accumulated in the presence of 5 mM proline, and large amounts of ammonia accumulated in the presence of glutamate as substrate. This suggests that, under this condition, glutamine synthetase was rate-limiting for glutamine synthesis; in that respect, it should be emphasized that the flux through glutamine synthetase represented a large fraction (471/805 = 58.5%) of the maximal capacity of glutamine synthetase when 5 mM glutamate was the substrate (see Table I for the rate of glutamine synthesis from glutamate and "Results" for the glutamine synthetase activity). The fact that large amounts of ammonia accumulated especially from alanine, asparagine, ornithine, and leucine and, to a lesser extent, from other amino acids suggests that the synthesis of glutamate from these amino acids was a limiting factor for glutamine synthesis. In that respect, the low rate of glutamine synthesis observed from aspartate and, to a lesser extent, from ornithine despite a large accumulation of both glutamate and ammonia is surprising. Note also that glucose represented the main carbon product of proline metabolism.

Effect of Metabolic Acidosis on Glutamine Synthesis from Alanine and Proline—To our knowledge, this study is the first to demonstrate that chronic metabolic acidosis causes a marked inhibition of glutamine synthetase activity in the renal cortex of the mouse. Thus, the renal cortex of the latter species differs from that of the rat, the other rodent species classically used in biological research, in which no change in glutamine synthetase activity was found in metabolic acidosis (4, 6, 7). The 75% inhibition of glutamine synthetase activity observed in the renal cortex of acidotic mice compared with the renal cortex of control mice was accompanied by an inhibition of glutamine synthesis from alanine and proline in the tubules from acidotic mice, which was on the same order of magnitude (70–86%) (Tables III and V). It is therefore very likely that inhibition of glutamine synthesis in the tubules from acidotic mice is fully explainable by the inhibition of glutamine synthetase activity that resulted mainly, if not completely, from the large decrease in the glutamine synthetase protein level shown in Fig. 2. The mechanism of such inhibition, which is clearly post-transcriptional because the glutamine synthetase mRNA levels remained unchanged in the renal cortex of acidotic mice, remains to be elucidated and deserves further study.

Theoretically, other possible explanations for the acidosis-induced reduction of glutamine synthesis would be an inhibi-



SCHEME 1. **Schematic representation of alanine and proline metabolism in isolated mouse renal proximal tubules.** This scheme shows that two alanine and two proline molecules are metabolized for each glutamine and glucose molecule synthesized. *ALAT*, alanine aminotransferase; *GLDH*, glutamate dehydrogenase; *GS*, glutamine synthetase; *PC*, pyruvate carboxylase; *CS*, citrate synthase; *PEPCK*, phosphoenolpyruvate carboxykinase; *PK*, pyruvate kinase; *TCA*, tricarboxylic acid. Note that intermediates of the tricarboxylic acid cycle and of the gluconeogenic pathway are omitted from this scheme.

tion of substrate utilization and/or a reduction of the provision of either glutamate or ammonia or both to the glutamine synthetase reaction. The data of Tables II–V clearly indicate that neither alanine nor proline removal was inhibited by metabolic acidosis. As a consequence, glutamate synthesis and availability from alanine and proline could not be diminished in tubules from acidotic mice (Scheme 1); in agreement with this view, substantial amounts of glutamate still accumulated in these tubules (Tables II–V). In addition, the calculated flux through glutamate dehydrogenase, which makes ammonia available for the glutamine synthetase reaction with both alanine and proline as substrate, was not reduced in tubules from acidotic mice compared with that in tubules from control mice (Tables III and V). Thus, inhibition of glutamine synthetase appears to be the only mechanism responsible for the inhibition of glutamine synthesis observed in this study.

Effect of Metabolic Acidosis on Ammonia Accumulation from Alanine and Proline—It is important to emphasize that ammonia accumulation was clearly and strikingly enhanced when proline (but not alanine) was the substrate. With both 0.5 and 1 mM proline as substrate, the large augmentation of ammonia accumulation in tubules from acidotic mice was essentially due to the inhibition of glutamine synthesis; indeed, inhibition of the synthesis of one glutamine molecule makes two nitrogen atoms available (Table IV). It should be emphasized that the stimulation of flux through glutamate dehydrogenase observed with 1 mM proline as substrate in tubules from acidotic mice (Table V) resulted from the inhibition of glutamine synthesis and not from an augmented activity of glutamate dehydrogenase. In agreement with this view are the following: (i) the absence of statistically significant stimulation of ammonia production via glutamate dehydrogenase in the absence of alanine and proline (Tables II and IV) and (ii) our findings that glutamate dehydrogenase activity in the renal cortex of acidotic mice was not stimulated compared with that in the renal cortex of control mice (data not shown).

Because the flux through glutamate dehydrogenase, which provides ammonia, was not diminished by acidosis, it may appear surprising at first sight that the inhibition of glutamine synthesis, which consumes ammonia, did not lead unequivocally to an augmented accumulation of ammonia when alanine was the substrate (Tables II and III). In fact, it is very likely that this apparent contradiction is due to experimental errors and biological variability that masked the expected increase in ammonia accumulation because both the values for glutamine synthesis from alanine in tubules from control mice and those for the inhibition of glutamine synthesis in tubules from acidotic animals were small in absolute terms (Table III).

Effect of Metabolic Acidosis on Glucose Synthesis from Alanine and Proline—Because metabolic acidosis did not alter alanine and proline utilization, but markedly inhibited glutamine synthesis by the tubules, it profoundly altered the metabolic pathways (Scheme 1) and the fate not only of the nitrogen as seen above, but also of the carbon skeleton of these two amino acids. As a matter of fact, metabolic acidosis stimulated glucose synthesis from 0.5 mM proline and 1 mM alanine and proline (Tables III–V). Such stimulation can be explained by a combination of the following mechanisms: (i) a 4-fold stimulation by metabolic acidosis of the phosphoenolpyruvate carboxykinase activity in the mouse renal cortex (n = four control mice and five acidotic mice) (data not shown), an effect classically observed in the rat kidney (see Ref. 31 for a review); (ii) with 1 mM alanine as substrate, by a diversion of the alanine-derived oxaloacetate from α-ketoglutarate and then glutamate and glutamine synthesis to glucose synthesis and complete oxidation (Table III and Scheme 1); and (iii) with 1 mM proline as substrate, by an increase in flux through glutamate dehydrogenase and therefore in the provision of oxaloacetate for glucose synthesis.

It is of interest to note that, although two alanine and two proline molecules are needed for the synthesis of one glucose molecule, glucose synthesis from 0.5 mM alanine was approximately half that observed with the same concentration of proline despite similar substrate utilization (Table III and V). As shown in Scheme 1 and calculated from the data presented in Tables III and V, this can be explained by the greater availability of oxaloacetate for glucose synthesis with proline compared with alanine as substrate. Indeed, the oxaloacetate synthesized from proline (taken as the flux through glutamate dehydrogenase) (Table V and Scheme 1) was greater than that synthesized from alanine (calculated as the difference between the alanine utilized minus the flux through glutamate dehydrogenase plus twice the glucose synthesized) (Table III and Scheme 1). In addition, all of the proline-derived oxaloacetate

was available for the phosphoenolpyruvate carboxykinase reaction; by contrast, a substantial fraction of the alanine-derived pyruvate had to be converted into the α-ketoglutarate needed for alanine transamination because of the combined action of pyruvate carboxylase, pyruvate dehydrogenase, citrate synthase, and the enzymes of the tricarboxylic acid cycle responsible for the synthesis of α-ketoglutarate from citrate. Therefore, with alanine as substrate, the citrate synthase reaction diverted substantial amounts of oxaloacetate from gluconeogenesis to citrate and then α-ketoglutarate, glutamate, and glutamine synthesis.

Effect of Metabolic Acidosis on Glutamine Metabolism—Our results suggest that, with respect to glutamine utilization and metabolism, the mouse renal proximal tubule responds to metabolic acidosis like the rat renal proximal tubule (see Ref. 32 for a recent review); indeed, metabolic acidosis was found to stimulate glutamine utilization and ammonia and glucose production as well as fluxes through glutaminase, glutamate dehydrogenase, and phosphoenolpyruvate carboxykinase (see Table VI and the corresponding comments). It should be emphasized that the stimulation of ammonia production in response to *in vivo* metabolic acidosis has previously been observed by Nagami *et al.* (33) in isolated mouse proximal tubules perfused *in vitro* with glutamine as substrate.

Physiological Significance—This study, which demonstrated that a 48-h metabolic acidosis strongly inhibited glutamine synthetase and glutamine synthesis from near-physiological concentrations of alanine and proline and markedly stimulated the accumulation of ammonia from proline, is of potential physiological importance. Indeed, such inhibition of glutamine synthesis, if it occurs like the inhibition of glutamine synthetase activity in the mouse kidney *in vivo*, would increase the ammonia available for the renal excretion of acid as ammonium ions and therefore would represent an important mechanism for maintaining the systemic acid-base balance. With respect to the acidosis-induced inhibition of glutamine synthetase activity, the mouse differs from the rat, although the kidneys of these two rodent species contain high activities of the opposing enzymes, glutamine synthetase and glutaminase (16, 34), and excrete urine with approximately neutral pH (4, 16).

To our knowledge, only glutamine uptake has so far been demonstrated in the mouse kidney *in vivo* (35). In this respect, the results of this study strongly suggest that the increased fluxes through glutaminase and glutamate dehydrogenase observed *in vitro* in response to *in vivo* metabolic acidosis also occur *in vivo*. By contrast, the occurrence of glutamine synthesis by the mouse kidney *in vivo* has not been demonstrated until now. Therefore, this leaves open the possibility that, *in vivo*, like in the rat kidney (34), glutamine degradation by glutaminase and glutamine synthesis by glutamine synthetase occur simultaneously in the mouse kidney. Similarly, it is unknown if the mouse kidney, like the human kidney (36–38), takes up alanine and proline from the circulating blood. If this is the case, then our results suggest that both alanine and proline might play an important role not only as regulators of renal ammoniagenesis, but also as energy providers for the renal cells and as glucose precursors.

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