

Effect of starvation on glutamine ammoniagenesis and gluconeogenesis in isolated mouse kidney tubules

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It has been shown recently that glutamine is taken up by the mouse kidney *in vivo*. However, knowledge about the fate of this amino acid and the regulation of its metabolism in the mouse kidney remains poor. Given the physiological and pathophysiological importance of renal glutamine metabolism and the increasing use of genetically modified mice in biological research, we have conducted a study to characterize glutamine metabolism in the mouse kidney. Proximal tubules isolated from fed and 48 h-starved mice and then incubated with a physiological concentration of glutamine, removed this amino acid and produced ammonium ions at similar rates. In agreement with this observation, activities of the ammoniagenic enzymes, glutaminase and glutamate dehydrogenase, were not different in the renal cortex of fed and starved mice, but the glutamate dehydrogenase mRNA level was elevated 4.5-fold in the renal cortex from starved mice. In contrast, glucose production from glutamine was greatly stimulated whereas the glutamine carbon removed, that was

presumably completely oxidized in tubules from fed mice, was virtually suppressed in tubules from starved animals. In accordance with the starvation-induced stimulation of glutamine gluconeogenesis, the activities and mRNA levels of glucose-6-phosphatase, and especially of phosphoenolpyruvate carboxykinase, but not of fructose-1,6-bisphosphatase, were increased in the renal cortex of starved mice. On the basis of our *in vitro* results, the elevated urinary excretion of ammonium ions observed in starved mice probably reflected an increased transport of these ions into the urine at the expense of those released into the renal veins rather than a stimulation of renal ammoniagenesis.

Key words: fructose-1,6-bisphosphatase, glucose-6-phosphatase, glutamate dehydrogenase, glutaminase, mRNAs, phosphoenolpyruvate carboxykinase.

INTRODUCTION

Glutamine is the most concentrated free amino acid in the blood of human and numerous other animal species, and it plays a central role in the inter-organ transport of carbon and nitrogen. This amino acid is also involved in a great number of metabolic pathways and physiological processes in various organs and tissues, including skeletal muscle, brain, liver, intestine, kidney, adipose tissue, lung and lymphocytes (for reviews, see [1,2]). Arteriovenous difference measurements have shown that, *in vivo* and under certain circumstances, the kidney of the mouse [3], like that of man and other animal species [4–6], takes up glutamine from the circulating blood. However, the metabolism of this substrate in the mouse kidney remains poorly characterized; to our knowledge, only one study has reported that glutamine is converted into glucose in mouse kidney proximal tubules [7].

In view of the well-established importance of glutamine as a precursor of the ammonium ions that are excreted in urine (for regulating systemic acid-base equilibrium), and also as an energy-provider and a precursor of glucose in the kidney [2,8,9], we considered it important to study the metabolism of glutamine in mouse kidney tissue *in vitro*. Another important reason that led us to conduct such a study is the development, over the last two decades, of hundreds of genetically modified mice, some of which may represent interesting models of human pathophysiology in which renal metabolism is altered. Given the importance of such models in modern biological research, the lack of information about the renal metabolism of the mouse, which may at first sight appear surprising, is probably due to technical difficulties related to the small size of mouse kidneys. Therefore, we decided to

characterize the metabolism of glutamine in mouse kidney cortex *in vitro* using miniaturized techniques. Since most of renal glutamine metabolism is known to occur in the proximal tubule [10], we prepared isolated mouse kidney proximal tubules for the present study. The tubules obtained were incubated with a physiological concentration of glutamine, and substrate uptake and product formation were measured in tubules from both fed and 48 h-starved mice. Adaptation of mouse renal glutamine metabolism to starvation was further characterized by measuring not only the activity of key enzymes of glutamine ammoniagenesis and gluconeogenesis, but also the renal level of the corresponding mRNAs.

For the first time, the results obtained provide information on both the fate of glutamine carbon and nitrogen in mouse tubules and on part of the biochemical and molecular mechanisms that are involved in the starvation-induced changes of this metabolism in the mouse kidney.

EXPERIMENTAL

Preparation of mouse kidney tubules

Female Swiss mice (OF1) (28–32 g) were obtained from IFFA-CREDO, Saint-Germain-sur-l'Arbresle, France. The animals had free access to water and were either fed on a standard diet (U.A.R., Villemoisson-sur-Orge, France) or starved for 48 h before the experiments. The mice were anaesthetized with intraperitoneal pentobarbital (50 mg/kg body weight), and then the kidneys were excised through a midline abdominal incision and placed in ice-cold Krebs–Henseleit medium. After the capsule had been removed, the cooled kidneys were longitudinally

Abbreviations used: P-enolpyruvate, phosphoenolpyruvate; RT, reverse transcriptase.

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hemisected and the medullary tissue was discarded. Slices of the cortex, made with a razor blade, were then incubated in a shaking water bath (40 cycles/min) for 15 min at 37 °C in a silicone-treated 25 ml Erlenmeyer flask, containing 3 ml of Krebs–Henseleit medium and collagenase (1.5 mg/ml) in an atmosphere of O₂/CO₂ (19:1). The resultant homogenate was gently forced through an 18-gauge needle to aid dispersion of the tubules, the undigested particles were removed using a tea strainer, and the filtrate was centrifuged at 40 *g* for 2 min at 4 °C. The supernatant containing kidney debris was carefully aspirated and discarded. The separated tubules were resuspended in 8 ml of cold Krebs–Henseleit medium and, after a second centrifugation, the final tubule pellet was resuspended in cold Krebs–Henseleit medium.

Incubations

Incubations were carried out in a shaking water bath in 12 ml stoppered Erlenmeyer flasks in an atmosphere of O₂/CO₂ (19:1). Tubules were incubated for 20–60 min in 1 ml of Krebs–Henseleit medium, with or without L-lactate or L-glutamine as substrate. The flasks were prepared in duplicate or triplicate for all experimental conditions. Incubations were terminated by adding HClO₄ (final concn. 3%, v/v). In all experiments, zero-time incubations, with and without substrate, were prepared by adding HClO₄ to the flasks before the tubules. After removal of the denaturated protein by centrifugation, the supernatant was neutralized with 20% (w/v) KOH for metabolite determination.

In two experiments designed to measure the glycogen content of the tubules, either at the start or at the end of the incubation period, the incubation medium was not acidified but cooled to 0 °C; the tubules were then separated from the medium by centrifugation and used for glycogen determination.

Urinary acid-base parameters

Four mice were placed in metabolic cages (Marty Techno, Marcilly s/ Eure, France) for 24 h in the fed state and during the second day of starvation, and their urine was collected under mineral oil. pH was measured with a Radiometer pH meter (PHM 62, Radiometer, Copenhagen, Denmark). Ammonia was measured in diluted samples of urine, and titratable acidity was measured by titrating suitably diluted samples of urine to pH 7.4 with 10 mM NaOH.

Analytical methods

Lactate, pyruvate, glucose, alanine, aspartate, citrate, 2-oxoglutarate, fumarate, malate, glutamate, glutamine, ammonia, ATP, glucose 6-phosphate and glycogen were determined by enzymic methods with a spectrofluorimeter (SLT FLUOstar, BMG, Offenburg, Germany) or spectrophotometer (Spectra-max®, Molecular Devices, Sunnyvale, CA, U.S.A.) [11]. The protein content of the tubules added to each flask was determined by the Lowry method [12].

Calculations

Net substrate utilization and product formation were calculated as the difference between the total contents of the flask (tissue + medium) at the start (zero-time incubations) and after various periods of incubation. The metabolic rates are expressed in nmol of substance removed or produced/h per mg tubule fragment protein and are given as means ± S.E.M. The results were analysed by the Student's *t* test for unpaired or paired data, comparing values obtained in fed mice with those in 48 h-starved mice.

Reagents

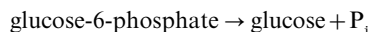
Glutaminase (grade V) was purchased from Sigma Chemical Co. (L'Isle d'Abeau Chesnes, France). Other enzymes, coenzymes and oligodT were obtained from Roche (Meylan, France). Superscript II™ reverse transcriptase (RT), platinum® Taq polymerase and dNTPs were obtained from Life Technologies (Pontoise, France). The mRNA extraction kit was purchased from Dynal (Oslo, Norway) and primers were obtained from Genset SA (Paris, France).

Measurement of enzyme activities

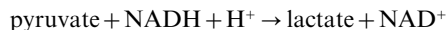
In the experimental groups used to measure enzyme activities or mRNA levels, the kidneys were removed, immediately frozen in liquid nitrogen, and kept at –80 °C until analysed. Kidney cortex homogenates were prepared at 0 °C in 20 mM phosphate buffer (pH 7.4), containing 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 25% (v/v) glycerol and 0.02% (w/v) BSA. The homogenates were diluted in an appropriate buffer for enzyme activity determinations. Measurement of glutaminase (EC 3.5.1.2) activity was performed at 37 °C, whereas measurements of glutamate dehydrogenase (EC 1.4.1.3), phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose-1,6-bisphosphatase (EC 3.1.3.11), glucose-6-phosphatase (EC 3.1.3.9) and pyruvate kinase (EC 2.7.1.40) activities were conducted at 25 °C. All the enzyme activities were measured according to the methods of Passonneau and Lowry [11]. The original description of these methods were reported by Burch et al. [13] for phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase, and by Curthoys and Lowry [14] for phosphate-activated glutaminase. However, since we used the spectrophotometer or fluorimeter microplate readers in the final measurement step, a full description of the methods employed is given below.

Glucose-6-phosphatase

The assay of this enzyme activity was conducted in two separate steps. In the first step, glucose 6-phosphate is hydrolysed by glucose-6-phosphatase:



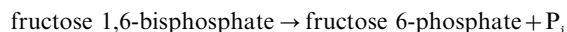
Then, the released glucose is converted back into glucose 6-phosphate by hexokinase in the presence of ATP, and the ADP formed was measured by the enzyme pathway given below:



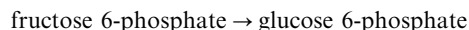
The reagent used in the first step was 100 mM imidazole-HCl buffer (pH 6.8) containing 1 mM EDTA, 0.02% (w/v) BSA and 15 mM glucose 6-phosphate. Aliquots (100 µl) of this reagent were placed in small glass tubes and 10 µl of dilute homogenate (30–50 µg tissue) was added. After incubation at 25 °C for 1 h, the tubes were heated for 2 min at 100 °C. Subsequently, 300 µl of the second step reagent [50 mM imidazole-HCl buffer (pH 7.2), 100 mM KCl, 150 µM ATP, 400 µM P-enolpyruvate, 2.5 mM MgCl₂, 2 mM 2-mercaptoethanol, 300 µM NADH, 0.4 unit/ml rabbit muscle pyruvate kinase, 2 units/ml beef heart lactate dehydrogenase] was added to each tube. Aliquots of the reaction mixture (350 µl) were transferred into a 96-well microtitre plate and the first reading was performed in the spectrophotometer at a wavelength of 340 nm. Then, 0.15 unit of hexokinase were added to each well and after 20 min, a second reading was performed. Glucose standards (0–60 nmoles) served as a standard and were processed in the same way as the samples.

Fructose-1,6-bisphosphatase

A direct kinetic fluorometric method was used, as described below. Hydrolysis of fructose 1,6-bisphosphate led to phosphate and fructose 6-phosphate:



The latter, used by phosphoglucose isomerase, provided glucose 6-phosphate:



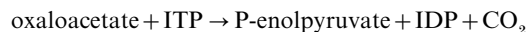
Glucose 6-phosphate and NADP⁺ were converted into 6-phosphogluconate and NADPH by glucose 6-phosphate dehydrogenase:



A 350 μl volume of reagent [50 mM imidazole-HCl buffer (pH 7), 1 mM EDTA, 0.02% (w/v) BSA, 2 mM 2-mercaptoethanol, 2 mM MgCl₂, 200 μM NADP⁺, 100 μM fructose 1,6-bisphosphate, 0.35 unit/ml yeast phosphoglucose isomerase and 0.25 unit/ml yeast glucose 6-phosphate dehydrogenase] was added to 10 μl of dilute homogenate (50–100 μg tissue) in a 96-well microtitre plate. The increase in fluorescence was followed for 10 min (excitation wavelength 350 nm and emission wavelength 460 nm). Fructose 6-phosphate standards (1.25–10 nmoles) were processed in the same way as the samples and were used to calibrate the fluorimeter.

P-enolpyruvate carboxykinase

This reaction was measured in the forward direction (P-enolpyruvate formation) and occurs in three steps:



For the first step, 50 μl of reagent [60 mM imidazole-sulphate (pH 7.2), 12 mM MgSO₄, 0.05% (w/v) BSA, 0.12 mM MnSO₄, 0.12 mM EGTA, 1.2 mM 2-mercaptoethanol, 6 mM ITP, 3 mM oxaloacetate] was mixed with 10 μl of dilute homogenate (1–2 μg tissue). After 1 h at 25 °C, the reaction was stopped by heating for 2 min at 100 °C. To destroy ketoacids, 5 μl of 3% (v/v) H₂O₂ was added, and the tubes were again boiled for 2 min. The addition of 10 μl of 400 units/ml beef liver catalase was used to remove H₂O₂. The whole content of each tube was transferred into a spectrophotometer 96-well microplate for a direct measurement of the accumulated P-enolpyruvate.

For the second step, 250 μl of reagent [100 mM imidazole-HCl buffer (pH 7.2), 300 μM ADP, 120 mM KCl, 200 μM NADH, 0.03% (w/v) BSA, 1 unit/ml beef heart lactate dehydrogenase] was added. A first reading was made at 340 nm and then 10 μl of 15 units/ml rabbit muscle pyruvate kinase was added. After 20 min at 23 °C, a second reading was taken. Standards of P-enolpyruvate (0–20 nmoles) were processed in the same way as homogenates.

Glutamate dehydrogenase

The activity was measured at 25 °C with NADPH as coenzyme in the direction of glutamate formation:

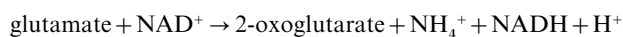
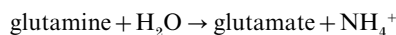


A volume of 300 μl of reagent [50 mM imidazole-HCl buffer (pH 7.0), 2 mM 2-oxoglutarate, 25 mM ammonium acetate,

100 μM ADP, 0.02% (w/v) BSA and 200 μM NADPH] was added to 50 μl of dilute homogenate (80 μg tissue) in a 96-well microtitre plate. The decrease in absorbance was followed directly using a spectrophotometer at 340 nm for 10 min.

Phosphate-activated glutaminase

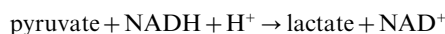
The assay was conducted in two separate steps:



In the first step, hydrolysis of glutamine was performed at 38 °C for 1 h. A 50 μl volume of reagent [75 mM Tris/HCl (pH 8.6), 30 mM glutamine, 150 mM phosphate (pH 8.0), 0.2 mM EDTA, 0.02% (w/v) BSA] was mixed with 10 μl (15–20 μg tissue) of homogenate. After 1 h, the assay was stopped with 10 μl of 0.8 M HCl. A 30 μl aliquot was transferred into a 96-well microtitre plate, and the accumulated glutamate was measured in a second step with NAD⁺ and glutamate dehydrogenase. After this, 350 μl of a second reagent [90 mM Tris/HCl (pH 8.4), 1 mM NAD⁺, 300 μM ADP, 0.03% (v/v) H₂O₂ and 6 units/ml beef liver glutamate dehydrogenase] was added. After 40 min, a spectrofluorimeter reading was made. Standards of glutamate (0–20 nmoles) were processed in the same way as samples.

Pyruvate kinase

Specific and auxiliary reactions were combined in a single step at 25 °C, and NADH consumption was followed in a spectrophotometer at 340 nm for 10 min.



A 30 μl aliquot of dilute homogenate (50–60 μg tissue) was mixed with 300 μl of reagent [100 mM imidazole/HCl (pH 7.0), 100 mM KCl, 3 mM MgCl₂, 1.5 mM P-enolpyruvate, 1 mM ADP, 300 μM NADH, 0.02% (w/v) BSA and 0.8 unit/ml beef heart lactate dehydrogenase] in a 96-well microtitre plate.

Semi-quantitative analysis of mRNA expression

To determine the glutamate dehydrogenase, phosphoenolpyruvate carboxykinase, glutaminase, glucose-6-phosphatase and fructose-1,6-bisphosphatase mRNA levels in fed and 48 h-starved mice kidney cortex, semi-quantitative RT-PCR was performed. Levels of each mRNA of interest were related to those of the housekeeping β -actin gene transcripts. Gene-specific oligonucleotide primers (20 nucleotides) were selected from the published cDNA sequences of mouse glutamate dehydrogenase, phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, fructose-1,6-bisphosphatase, and from the cDNA sequence of rat mitochondrial kidney-type glutaminase. As an internal control, a primer pair was selected from the cDNA sequence of mouse β -actin. Forward and reverse primers chosen for glutaminase, glutamate dehydrogenase, fructose-1,6-bisphosphatase, glucose-6-phosphatase, phosphoenolpyruvate carboxykinase and β -actin are shown in Table 1.

The use of the β -actin gene as an internal control provided a standard by which differences in the expression levels, owing to underestimation or overestimation of the input amount of the RNA template, could be normalized. PCR products of the expected size were sequenced by Genome Express (Grenoble, France). The sequences for glutamate dehydrogenase, phosphoenolpyruvate carboxykinase, glucose-6-phosphatase and fructose-1,6-bisphosphatase were found to be identical to the

Table 1 Parameters of the PCR analysis

β -Actin cDNA and each enzyme cDNA were co-amplified in the same tube.

Protein												
Parameter	Glutaminase	Glutamate dehydrogenase	Fructose-1,6-bisphosphatase	Glucose-6-phosphatase	P-enolpyruvate carboxykinase	β -Actin						
Bank accession no.	NM_012569	NM_008133	NM_007994	NM_008061	NM_011044	X03672						
Primers												
Forward	5'-GACTTCTCAGGGCAGTTTG-3'	5'-GAGCGCTCTCCTTCCCATCC-3'	5'-CCCTCTGGATGGATCTTCAA-3'	5'-TCTCTTTCCCATCTGGTTC-3'	5'-AGGATCGAAAGACAGACAGT-3'	5'-CTGGCACCACACCTTCTACA-3'						
Reverse	5'-TGTCACAAAGTGCAGTGTTC-3'	5'-ATAGTGCCCTATGGTGTGG-3'	5'-CTGTGGTTGCCATACCTCT-3'	5'-TATACACCTGCTGGCCCAT-3'	5'-TGGGATGACATACATGGTGC-3'	5'-AGTACTTGGGCTCAGGAGGA-3'						
PCR product length (bp)	497	394	539	247	171	758						
Specific segments (nt)	1472-1968	424-817	376-914	216-462	399-569	335-1092						
PCR parameters	23 cycles 30 s at 94 °C 45 s at 60 °C 1 min at 72 °C	23 cycles 30 s at 94 °C 45 s at 60 °C 1 min at 72 °C	22 cycles 30 s at 94 °C 45 s at 60 °C 1 min at 72 °C	20 cycles 30 s at 94 °C 45 s at 60 °C 1 min at 72 °C	18 cycles 30 s at 94 °C 45 s at 60 °C 1 min at 72 °C	18 cycles 30 s at 94 °C 45 s at 60 °C 1 min at 72 °C						

published mouse cDNAs. Alignment of the amplified glutaminase sequence with the published rat kidney glutaminase cDNA using BLAST at the National Center for Biotechnology Information (Bethesda, MD, U.S.A.) showed more than 93 % identity. mRNA was extracted using Dynabeads[®] mRNA Direct[™] kit, (Dyna[®], Oslo) and about 200 ng of mRNA was reverse-transcribed for 1 h at 42 °C with non-specific oligo(dT)₁₅₋₁₈, using 600 units of superscript[™] II RT in a total volume of 75 μ l. Control reactions did not contain RT.

A 5 μ l cDNA aliquot was then subjected to PCR amplification with specific primers for β -actin and for the five enzymes studied. The specific primers for the enzymes were included in the reaction mix at the beginning of the amplification step. Depending on the enzyme studied (see Table 1), the specific primers for β -actin were added to each tube after 2, 4 or 5 cycles of amplification, so that the β -actin cDNA was co-amplified with the cDNA of interest. Preliminary experiments served to establish the linearity of amplification. All PCR reactions were performed in a thermocycler (Mastercycler[®] Personal, Eppendorf) with 2 mM MgCl₂, 0.4 μ M of each primer, 0.3 mM dNTPs and 1.3 units of platinum[®] Taq polymerase in a total volume of 50 μ l. The PCR programs are presented in Table 1.

PCR products were separated electrophoretically on a 1.7 % (w/v) agarose gel and stained with SYBR[®] Green I (Molecular Probes Europe, Leiden, The Netherlands). The gel was then scanned using a fluorescence laser scanner (Molecular Dynamics, SA, U.S.A.). Band intensities were quantified using the Image Quant[®] software. mRNA levels are reported relative to β -actin.

RESULTS

Time course of lactate and glutamine metabolism in isolated mouse kidney tubules

Figure 1 shows the results of two representative experiments (one with tubules from one fed mouse, and one with tubules from a starved mouse) designed to test the metabolic viability of mouse

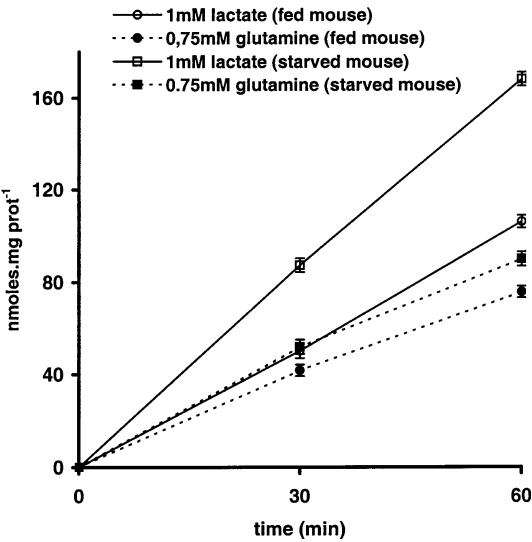


Figure 1 Time course of glucose production from 1 mM lactate and 0.75 mM glutamine in isolated mouse kidney tubules

Values are means \pm S.E.M. for triplicate conditions at each time point in two representative experiments. The flasks contained 0.64 mg protein (fed mouse) and 0.60 mg protein (48 h-starved mouse).

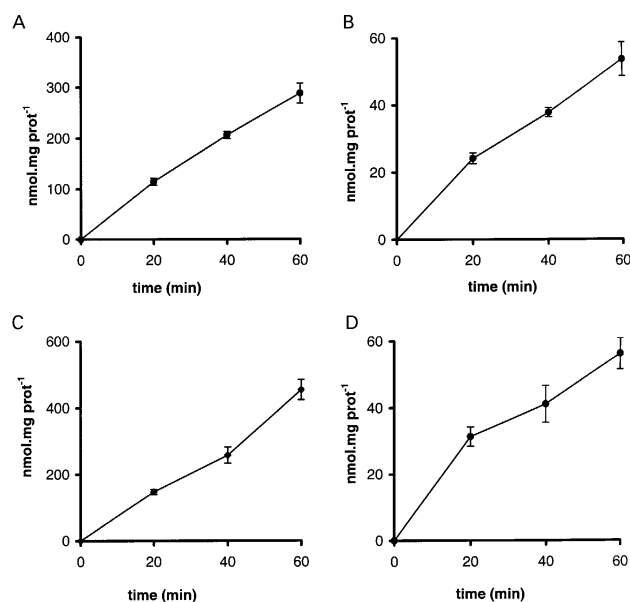


Figure 2 Time course of glutamine utilization (A), glucose (B) and ammonia (C) production, and glutamate accumulation (D) in isolated mouse kidney tubules

Values are means \pm S.E.M. for triplicate conditions at each time point in a representative experiment performed with kidney tubules from one fed mouse. The flasks contained 0.74 mg protein.

Table 2 Metabolism of lactate and glutamine in kidney tubules from fed and 48 h-starved mice

Kidney tubules (0.61 ± 0.06 and 0.71 ± 0.05 mg protein per flask for fed and starved mice respectively) were incubated as described in the Experimental section. Results (nmol \cdot mg protein $^{-1} \cdot$ h $^{-1}$) for metabolite removal (—) or production are reported as means \pm S.E.M. for seven and six experiments performed in triplicate in fed and starved mice respectively. Statistical difference between the fed and starved state was measured by the unpaired Student's *t* test (**P* < 0.05).

Nutritional state	Metabolite removal or production					
	Lactate (1 mM)		Glutamine (0.75 mM)			
	Lactate	Glucose	Glutamine	Glutamate	Glucose	NH $_4^+$
Fed	-598 ± 51	122 ± 9	-287 ± 27	57 ± 3	81 ± 6	562 ± 85
Starved	-576 ± 52	$283 \pm 32^*$	-262 ± 38	$81 \pm 8^*$	$108 \pm 7^*$	546 ± 53

kidney tubules. It can be seen that glucose synthesis from a physiological concentration of lactate (1 mM) or glutamine (0.75 mM), two major substrates taken up from circulating blood by the kidney of various species, was linear with time for up to 60 min of incubation of kidney tubules either from fed or starved mice; the rate of glucose synthesis was found to decline for longer incubation periods (results not shown). Addition of 1μ M adrenaline to the incubation medium of tubules metabolizing lactate (1 mM) led to a 20% stimulation of gluconeogenesis ($n = 5$, $P < 0.05$; results not shown). Figure 2 shows that mouse kidney tubules also removed glutamine and produced glucose and ammonia in a linear manner for up to 60 min of incubation. Although this was not the case in all the experiments performed, glutamate, which is an intermediate and not an end-product of glutamine metabolism, was also found to

accumulate in a linear manner with time, the results of the representative experiment are given in Figure 2.

With lactate or glutamine as substrate, the ATP concentration in kidney tubules from fed ($n = 2$) and starved ($n = 2$) mice was 10.1 ± 0.7 and 16.9 ± 0.8 nmol/mg of protein at the start and the end of the incubation period respectively. Taking a protein amount of 20% of fresh weight for the tubules, the ATP value found at the end of the incubation period corresponded to 3.4μ M \cdot g tubular fresh weight $^{-1}$, which is virtually identical to that measured in the extract of a freeze-clamped kidney from a fed mouse (3.3μ M \cdot g fresh weight $^{-1}$).

Effect of starvation on glutamine metabolism in isolated mouse kidney tubules

Table 2 shows the results of two experimental series aimed at studying the influence of a 48 h-starvation on the removal of 1 mM lactate and its conversion into glucose, as well as on the metabolism of 0.75 mM glutamine in mouse kidney tubules. Starvation did not alter the removal of lactate, whereas it caused a 132% stimulation of glucose production from lactate. Assuming that, in the presence of lactate, all the glucose found was derived from lactate, and given that two lactate molecules are needed to synthesize one glucose molecule, carbon balance calculations indicate that glucose accounted for 41% of the lactate removed in tubules from fed mice and for 98% in tubules from starved mice.

Table 2 also shows that starvation did not change glutamine removal and ammonia production but significantly increased glutamate accumulation by 40% and glucose production by 33%. Since two glutamine molecules are needed to synthesize one glucose molecule, it can be calculated that glucose accounted for 56% and 82% of the glutamine carbon removed by kidney tubules from fed and starved mice respectively. Again, the latter calculations are based on the assumption that all the glucose found came from glutamine. In the absence of exogenous substrate, the production of glucose was 21 ± 2.1 and 40 ± 4.3 ($P < 0.05$) nmol \cdot mg of protein $^{-1} \cdot$ h $^{-1}$ in tubules from fed and starved animals respectively. Tubules from fed mice contained 7 nmol \cdot mg of protein $^{-1}$ of glycogen (expressed in glucosyl units) at the start of incubation, both in the absence and presence of substrates, and this value remained unchanged at the end of the incubation period ($n = 2$). Under all experimental conditions, glucose 6-phosphate was undetectable ($n = 2$). No substantial amounts of alanine, aspartate, pyruvate, lactate or intermediates of the tricarboxylic acid cycle were found to accumulate with glutamine as substrate. The data in Table 2 also reveal that, at physiological concentrations, lactate appears to be a better gluconeogenic precursor than glutamine in tubules from both fed and starved mice ($P < 0.05$ in both nutritional states).

Considering that each glutamine molecule contains two nitrogen atoms, nitrogen balance calculations indicate that the nitrogen found as ammonia and glutamate exceeded that removed as glutamine. This means that some of the ammonia found in the presence of glutamine arose from endogenous substrates. In this respect, the production of ammonia in the absence of exogenous substrate was 86 ± 24 and 100 ± 24 nmol \cdot mg of protein $^{-1} \cdot$ h $^{-1}$ in tubules from fed and starved mice respectively. These values were not statistically different.

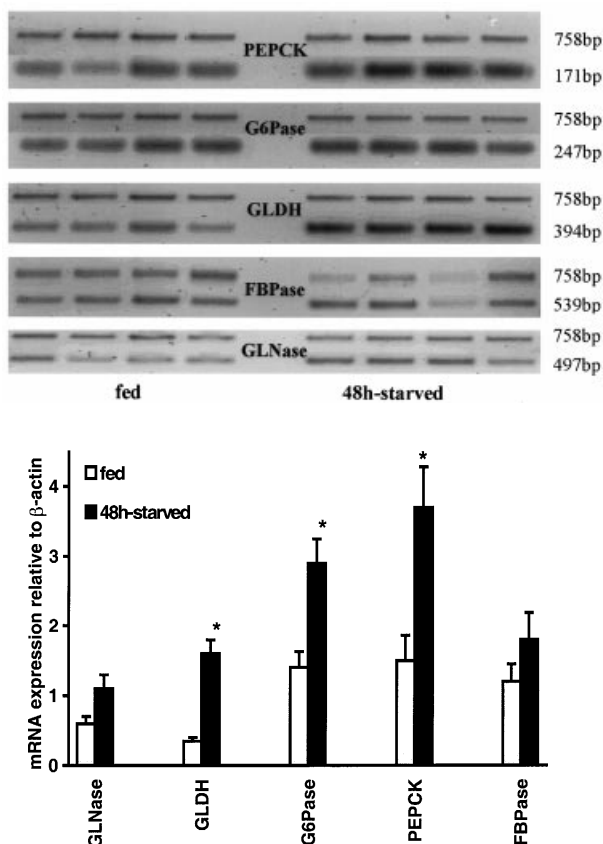
Effect of starvation on the activity of key enzymes of ammoniogenesis and gluconeogenesis from glutamine and on the corresponding mRNA levels

Table 3 shows that starvation significantly diminished the activity of glutaminase by 22% but did not change that of glutamate

Table 3 Activity of key enzymes of glutamine ammoniogenesis and gluconeogenesis in the renal cortex of fed and 48 h-starved mice

Values, expressed in $\mu\text{mol} \cdot \text{mg of protein}^{-1} \cdot \text{h}^{-1}$, are given as means \pm S.E.M. for nine fed and nine starved mice. Statistical difference between the fed and starved state was measured by the unpaired Student's *t* test (**P* < 0.05).

Nutritional state	Glutaminase	Glutamate dehydrogenase	P-enolpyruvate carboxykinase	Fructose-1,6-bisphosphatase	Glucose-6-phosphatase	Pyruvate kinase
Fed	1.8 \pm 0.1	1.5 \pm 0.1	1.3 \pm 0.1	3.8 \pm 0.1	3.5 \pm 0.2	7.7 \pm 0.3
Starved	1.4 \pm 0.1*	1.6 \pm 0.1	6.8 \pm 0.6*	3.6 \pm 0.2	4.9 \pm 0.4*	7.2 \pm 0.3

**Figure 3 Cortical mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), glutamate dehydrogenase (GLDH), fructose-1,6-bisphosphatase (FBPase), glutaminase (GLNase) and β -actin (758 bp) in four fed and four 48 h-starved mice**

The mRNA levels were analysed by semi-quantitative RT-PCR analysis, as described in the Experimental section. The amplified cDNAs were separated by agarose-gel electrophoresis, as shown in the upper part of the figure (left lanes: fed mice, right lanes: starved mice). Band intensities were quantified and are reported relative to the β -actin band (lower part of the figure). The values are means \pm S.E.M. for four fed and four 48 h-starved mice. The statistical difference between fed and starved mice was measured by the unpaired Student's *t* test (**P* < 0.05).

dehydrogenase. Among the three key gluconeogenic enzymes involved in glutamine metabolism, starvation induced a 443% and 38% increase in the activity of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, respectively; by contrast, the activities of fructose-1,6-bisphosphatase and pyruvate kinase were not altered by starvation.

As can be seen in Figure 3, co-amplification of enzyme cDNA with β -actin cDNA revealed that starvation did not reduce the cellular level of glutaminase mRNA, nor modified that of fructose-1,6-bisphosphatase mRNA. On the contrary, a

Table 4 Effect of starvation on the urinary excretion of total acid, titratable acid, ammonium ions, and on urine pH in mice

Values are expressed as means \pm S.E.M. for four mice weighing initially 31.5 ± 1.9 g. The excretion of total acid, titratable acid and ammonium ions is given in $\mu\text{mol} \cdot 24 \text{ h}^{-1}$. In starved mice, the urine was collected during the second day of starvation. Statistical difference between the fed and starved state was measured by the paired Student's *t* test (**P* < 0.05).

Nutritional state	Total acid	Titratable acid	Ammonium ions	Urine pH
Fed	163 \pm 18	74 \pm 11	89 \pm 10	6.52 \pm 0.21
Starved	320 \pm 57*	135 \pm 27	185 \pm 34*	5.72 \pm 0.03*

significant elevation of the cellular levels of glutamate dehydrogenase (4.5-fold increase), phosphoenolpyruvate carboxykinase (2.5-fold increase) and glucose-6-phosphatase (2-fold increase) mRNAs occurred in the starved state.

Effect of starvation on the urinary acid excretion

Table 4 shows the values of the urinary acid-base parameters measured. It can be seen that, during the second day of starvation, a fall of urinary pH and a statistically significant increase in the excretion of acid (ammonium ions plus titratable acid) were observed. The urinary excretion of ammonium ions almost doubled, whereas that of titratable acid increased in a manner that was at the borderline of statistical significance (*P* = 0.059).

DISCUSSION

To our knowledge, this study represents the first attempt to offer an integrated view on the changes that occur in glutamine metabolism in kidney tubules from starved mice compared with fed mice. We studied simultaneously (i) changes in fluxes through enzymes responsible for the metabolism of a physiological concentration of glutamine in isolated kidney tubules, (ii) changes in the activity of key enzymes involved in glutamine ammoniogenesis and gluconeogenesis and (iii) changes in the corresponding mRNA levels.

Metabolic viability of mouse kidney tubules

The linearity with time of the synthesis of glucose from both lactate and glutamine indicates that our isolated mouse kidney tubules were metabolically viable. Indeed, gluconeogenesis from these substrates requires co-ordination between the transport of these substrates and the operation of a great number of enzymes, together with the functional integrity of mitochondria. It is worthy of note that the rate of gluconeogenesis from 0.75 mM glutamine in our mouse tubules was severalfold greater than that from 1 mM glutamine in mouse tubules prepared by other workers [7]. The stimulatory effect of adrenaline on lactate gluconeogenesis in these tubules, like in isolated rat kidney

tubules [15], represents a further indication of their functional integrity. Moreover, the fact that the ATP concentration in our incubated mouse tubules was found to be similar to that measured in the mouse kidney *in vivo* strongly suggests that virtually all the cells of our tubules were viable.

Effect of starvation on the metabolic fate of glutamine carbon and nitrogen

The fact that neither glutamine removal nor ammonia production significantly changed in tubules from starved mice when compared with tubules from fed animals, indicates that fluxes through glutaminase and glutamate dehydrogenase, the two ammonia-genic enzymes involved in renal glutamine metabolism, were not modified by starvation. In this respect, mouse kidney tubules resemble rat kidney cortex slices [16]. It is also important to note that carbon balance calculations reveal that 76% and 100% of the glutamine carbon skeleton removed was accounted for by the non-volatile carbon products (glutamate and glucose) found to accumulate in tubules from fed and starved mice respectively. Although we have not measured the production of CO₂ from glutamine in this study, these data strongly suggest that starvation suppressed the complete oxidation of glutamine, which represented about one-quarter of the glutamine removed in fed mice. Thus, it seems that stimulation of the conversion of glutamine into glucose in kidney tubules from starved mice occurs at the expense of complete oxidation.

Regulatory steps of glutamine metabolism and adaptation to starvation

After correcting for a temperature of 37 °C (except in the case of glutaminase) by multiplying by 2 the values presented in Table 3, it is interesting to compare the capacities of the key enzymes of glutamine metabolism with fluxes through these enzymes in intact tubules metabolizing glutamine (see data in Table 2 for the calculations of enzymic fluxes). It appears that fluxes through glutaminase (taken as the removal of glutamine), through glutamate dehydrogenase and phosphoenolpyruvate carboxykinase (taken as the difference between glutamine removal and glutamate accumulation), through fructose-1,6-bisphosphatase and glucose-6-phosphatase (taken as the production of glucose) were always much lower than the corresponding capacities; indeed, in tubules from fed and starved mice, these fluxes represented 16% and 19% of glutaminase capacity, 8% and 6% of glutamate dehydrogenase capacity, and 9% and 1% of phosphoenolpyruvate carboxykinase capacity respectively. Fluxes through fructose-1,6-bisphosphatase and glucose-6-phosphatase represented less than 2% of the corresponding capacities in both the fed and starved states. Since the synthesis of oxaloacetate from glutamine, taken as the flux through glutamate dehydrogenase, was not altered by starvation ($P = 0.09$), it is very likely that, under these conditions, the large increase in the phosphoenolpyruvate carboxykinase activity (without any change in the pyruvate kinase activity) shown in Table 3, played a major role in the mechanism responsible for the increased conversion of glutamine carbon into glucose. The smaller starvation-induced increase in glucose-6-phosphatase activity suggests that the latter enzyme does not play a central role in the adaptation to starvation of glucose synthesis from glutamine in the mouse kidney. It should be noted that an enhanced activity of phosphoenolpyruvate carboxykinase, but not of glucose-6-phosphatase, has been reported by other workers in the kidney of mice starved for 48 h [17]. An elevated level of phosphoenolpyruvate carboxykinase mRNA has also been observed in the

kidney of starved mice [18]. Furthermore, augmented phosphoenolpyruvate carboxykinase and glucose-6-phosphatase activities and/or mRNA levels have been observed previously in the kidney of starved rats [13,19–22].

It should also be noted that the decrease in the capacity of glutaminase caused by starvation (Table 3) was not accompanied by a diminution of flux through the latter enzyme (see Table 2).

It is also interesting to compare the changes caused by starvation in the activities of the key enzymes studied and in their corresponding mRNA levels. It might be expected that increases in the phosphoenolpyruvate carboxykinase and glucose-6-phosphatase mRNA levels would be in the same ranges as the corresponding enzyme activities. However, there was no correlation between the large starvation-induced elevation of glutamate dehydrogenase mRNA level and the corresponding enzyme activity or flux, which did not change. In view of the time lag demonstrated in the rat kidney during short-term metabolic acidosis (compared with metabolic alkalosis) between the 3.5-fold increase in the glutamate dehydrogenase mRNA level at one day and the 16% increase in enzyme activity at only two days [23], we tested if a time lag would be observed in the mouse kidney between the induction of the transcript and that of the enzyme activity. For this, we measured both the glutamate dehydrogenase activity and the mRNA level in the renal cortex of four 72 h-starved mice. Confirming the results obtained after 48 h of starvation (Table 3 and Figure 3), glutamate dehydrogenase activity remained unchanged when compared with that measured in the renal cortex of fed mice, despite a 3.8-fold elevation of the glutamate dehydrogenase mRNA level (results not shown). In the same experiments, glutaminase activity was found to be decreased by 30% ($P < 0.05$), whereas the glutaminase mRNA level did not change (results not shown). Given the good correlations found in the rat kidney during chronic metabolic acidosis [14,23,24] between the activities and mRNA levels of glutamate dehydrogenase and/or glutaminase, the lack of correlation found in the present study in the mouse renal cortex may appear surprising (although not all studies have reported good correlations [25]). To our knowledge, such correlations have never been studied in the rat kidney during starvation. Therefore it is conceivable that the molecular mechanisms controlling the glutamate dehydrogenase and glutaminase mRNA levels and activities during starvation are different from those occurring during chronic metabolic acidosis.

In the rat kidney or in cultured renal cells, it is considered that alteration in renal phosphoenolpyruvate carboxykinase mRNA level by nutritional, hormonal or acid-base manipulations *in vivo* or *in vitro* is primarily due to a change in the rate of transcription of the corresponding gene [26,27]; the inhibition by actinomycin D of the starvation-induced elevation of phosphoenolpyruvate carboxykinase activity in the rat kidney [20] strongly suggests that an increased transcription of the phosphoenolpyruvate carboxykinase gene occurred in the kidney of our starved mice. The mechanism (the stimulation of the rate of transcription or stabilization of the mRNAs) responsible for the increased glutamate dehydrogenase and glucose-6-phosphatase mRNA levels that we observed merits further study. In this respect, it should be mentioned that experiments performed in rats *in vivo*, and with LLC-PK-F⁺ cells, indicates that changes in renal glutamate dehydrogenase mRNA levels in response to metabolic acidosis result from stabilization of these mRNAs [28].

Physiological significance

It is interesting to compare the rates of glutamine uptake found in the present study with that recently reported by Hallemeech

