Complexity of glutamine metabolism in kidney tubules from fed and fasted rats

Barbara VERCOUTÈRE, Daniel DUROZARD, Gabriel BAVEREL and Guy MARTIN

Centre d'Etudes Métaboliques par Spectroscopie de Résonance Magnétique (INSERM U499), Pavillon P, Hôpital Edouard Herriot, place d'Arsonval, 69374 Lyon Cedex 03, France

Glutamine is an important renal glucose precursor and energy provider. In order to advance our understanding of the underlying metabolic processes, we studied the metabolism of variously labelled [^{13}C]glutamine and [^{14}C]glutamine molecules and the effects of fasting in isolated rat renal proximal tubules. Absolute fluxes through the enzymes involved, including enzymes of four different cycles operating concomitantly, were assessed by combining mainly the ^{13}C NMR data with an appropriate model of glutamine metabolism. In both nutritional states, unidirectional glutamine removal by glutaminase was partially masked by the concomitant operation of glutamine synthetase; fasting accelerated glutamine removal by increasing flux solely through glutaminase, without changing that through glutamine synthetase. Fasting stimulated net glutamate degradation only by decreasing flux through glutamate dehydrogenase in the reductive amination direction, but surprisingly did not significantly alter complete oxidation of the glutamine carbon skeleton. Finally, gluconeogenesis from glutamine involved not only substantial recycling through the tricarboxylic acid cycle, but also an important anaplerotic flux through pyruvate carboxylase that was accelerated dramatically by fasting. Thus renal glutamine metabolism follows an unexpectedly complex route that is precisely regulated during fasting.

Key words: ammoniagenesis, ^{13}C NMR, gluconeogenesis, glutamine metabolism, kidney tubule, pyruvate carboxylase.

INTRODUCTION

Glutamine, the most highly concentrated free amino acid in the blood of humans and numerous animal species, is involved in a great variety of metabolic pathways and plays key roles in many physiological processes in various organs (see [1–3] for reviews). In the kidney, glutamine is the precursor of most of the ammonium ions that are excreted in the urine to protect the systemic acid–base balance [4,5]. Thanks to the partial and especially the complete oxidation of its carbon skeleton, glutamine is also an energy provider for renal reabsorptive work [6]. The contribution of glutamine to human or animal renal gluconeogenesis *in vivo* remains controversial and uncertain because the chemical or isotopic arteriovenous differences measured for glucose were within the margins of error of the analytical methods employed; despite this, it has been suggested mainly in recent studies that under certain conditions renal gluconeogenesis may represent a significant fraction (5–45 %) of systemic glucose production in humans [2,7–10].

Most arteriovenous difference measurements have shown that, unlike the kidneys of humans, dogs and monkeys, which extract significant amounts of circulating glutamine [11–15], the kidney of the rat takes up or releases very little glutamine under normal conditions *in vivo* [4,16–22]. This may at first sight appear surprising, since the rat kidney contains a high activity of glutaminase, the enzyme that initiates the degradation of glutamine [23]. In fact, isotopic tracer studies performed in the rat kidney *in vivo* [24] showed that, under normal acid–base conditions, flux through glutaminase was virtually equal to that through glutamine synthetase, the enzyme responsible for glutamine synthesis, which is also active in the rat kidney. In contrast, large amounts of glutamine are extracted by the kidneys of rats rendered acidotic or diabetic, or fed a high-casein diet or starved for 72 h [4,16,17,19–22,25]. Thus, under all experimental conditions and even when flux through glutaminase is masked by that through glutamine synthetase, glutamine appears to be a substrate that is taken up unidirectionally by the rat kidney.

A number of studies performed *ex vivo* with the isolated perfused rat kidney or *in vitro* with rat kidney-cortex slices or tubules have examined the fate of glutamine and the pathways involved (see for example [26–33]). They have shown that, depending on the nutritional state and acid–base conditions, the glutamine carbons are converted in various proportions mainly into the end-products glucose and carbon dioxide [26–33]. However, given the potential importance of, and the renewed interest in, renal gluconeogenesis from glutamine, especially in the fasted state, we felt that it was especially important to re-examine and characterize thoroughly this metabolic process in both the fed and the fasted states. For this, we have incubated isolated kidney tubules in the presence of ^{13}C-labelled and ^{14}C-labelled glutamine, and have taken advantage of the use of ^{13}C NMR spectroscopy, which has allowed us to unravel the complexity of the renal metabolism of various substrates in previous studies from this laboratory [34–37]. With the data obtained, which were combined with a model of glutamate and glutamine metabolism developed previously [38], we were able to estimate the fluxes through enzymes of glutamine metabolism in kidney tubules from fed and fasted rats.

EXPERIMENTAL

Reagents

Glutaminase (grade V) was from Sigma. Other enzymes and coenzymes were purchased from Boehringer Mannheim (Meylan, France). L-[1-^{14}C]Glutamate (1.85 GBq/mmol) and L-[1,5-^{14}C]glutamate (2.15 GBq/mmol) were supplied by Amersham Corp. (Les Ulis, France). L-[1-^{14}C]Glutamine and L-[1,5-^{14}C]glutamine

Abbreviation used: TCA cycle, tricarboxylic acid cycle.

[1] To whom correspondence should be addressed (e-mail baverel@laennec.univ-lyon1.fr).

were synthesized as described previously [26]. L-[1,2-^{13}C]Glutamine, L-[3-^{13}C]glutamine and $NaH^{13}CO_3$ were obtained from Euriso-Top (St Aubain, France). These ^{13}C-labelled compounds had an isotopic abundance of 99 %.

Rats

Male Wistar rats (260–280 g) were obtained from Iffa-Credo (Saint Germain sur l'Arbresle, France). They were fed a standard diet (U. A. R., Villemoisson-sur-Orge, France) or fasted for 48 h.

Preparation of kidney tubules and incubations

Kidney-cortex tubules were prepared by collagenase treatment of renal cortex slices as described by Baverel et al. [39]. Our method was also used by Vinay et al. [40], who thoroughly characterized the suspensions of tubules obtained. Considering that approx. two-thirds of the rat renal cortex comprises proximal tubules [41], it can be calculated on the basis of the phosphoenolpyruvate carboxykinase activity (which is exclusively proximal [42–44]) measured by Vinay et al. [40] in rat renal cortex homogenates and in rat kidney-cortex tubule suspensions that the latter preparations contained > 95 % proximal tubules. Incubations other than those involving radioactive substrates were performed for 60 min at 37 °C in a shaking water bath, in 25-ml stoppered Erlenmeyer flasks in an atmosphere of O_2/CO_2 (19:1). The flasks contained 1 ml of the tubule suspension plus 3 ml of Krebs–Henseleit medium [45] supplemented or not with substrates, i.e. 5 mM (final concentration) L-[1,5-^{14}C]glutamine (4×10^3 Bq/flask), L-[1-^{14}C]glutamine (2×10^3 Bq/flask), L-[1,2-^{13}C]glutamine, L-[3-^{13}C]glutamine or unlabelled glutamine plus 25 mM $NaH^{13}CO_3$. These differently labelled glutamines were used in an attempt to define the fate of all of the glutamine carbons, which depends on the metabolic pathways involved. Moreover, ^{13}C-labelled bicarbonate was used to measure the pyruvate carboxylase-mediated incorporation of the bicarbonate carbon into C-1 of glutamate, glutamine and serine, and C-3 and C-4 of glucose. In each experiment, each condition was performed in quadruplicate using a pool of tubules prepared from four kidney cortices from two rats. Incubations were stopped by adding $HClO_4$ (perchloric acid; final concentration 2 %, v/v) to each flask. Metabolite assays were conducted on the neutralized supernatant. In all experiments, zero-time flasks, with and without substrates, were prepared by adding $HClO_4$ immediately after the tubules. When radioactive glutamine was present in the medium, incubation, deproteination, collection and measurement of the $^{14}CO_2$ formed were performed as described by Baverel and Lund [26]. After removal of the denatured protein by centrifugation, the supernatant was neutralized with a mixture of 20 % (w/v) KOH and 1 % (v/v) H_3PO_4 for metabolite determination and NMR spectroscopy measurements.

Analytical methods

Metabolite assays

Glucose, glycogen, lactate, pyruvate, glutamate, glutamine, alanine, aspartate, citrate, 2-oxoglutarate, fumarate, malate, acetate, acetoacetate and 3-hydroxybutyrate, as well as the dry weight of tubules added to the flasks, were determined as described previously [26,39].

^{13}C NMR techniques

Data were recorded as indicated previously [34,35] at 125.75 MHz on a Bruker AM-500 WB spectrometer using a 10 mm broadband probe thermostatically controlled at 8 ± 0.5 °C. In brief, magnet homogeneity was adjusted using the deuterium lock signal. Supernatants, obtained from four flasks for each experimental condition with L-[1,2-^{13}C]glutamine, L-[3-^{13}C]glutamine or $NaH^{13}CO_3$ plus unlabelled glutamine as substrates, were pooled and freeze-dried; the resulting material was redissolved in ^2H_2O containing [2-^{13}C]glycine as internal standard, and centrifuged (5000 ***g***, 4 °C, 15 min). In order to obtain absolute quantitative results, special care was taken in data acquisition.

Acquisition parameters were as follows: spectral width, 25 000 Hz; tilt angle, 90°; data size, 32K; repetition time, 50 s; number of scans, 600. We used a standard (Waltz-16) pulse sequence for inverse-gated proton decoupling [46]. We did not use nuclear Overhauser enhancement during proton decoupling to avoid the use of corresponding correction factors. A 1 Hz line broadening was applied prior to Fourier transformation. Peak areas were determined by measuring the integral heights. Chemical shifts were expressed as p.p.m. relative to tetramethylsilane. Assignments were made by comparing the chemical shifts obtained with those given in the literature [47,48].

Calculations and statistical analysis

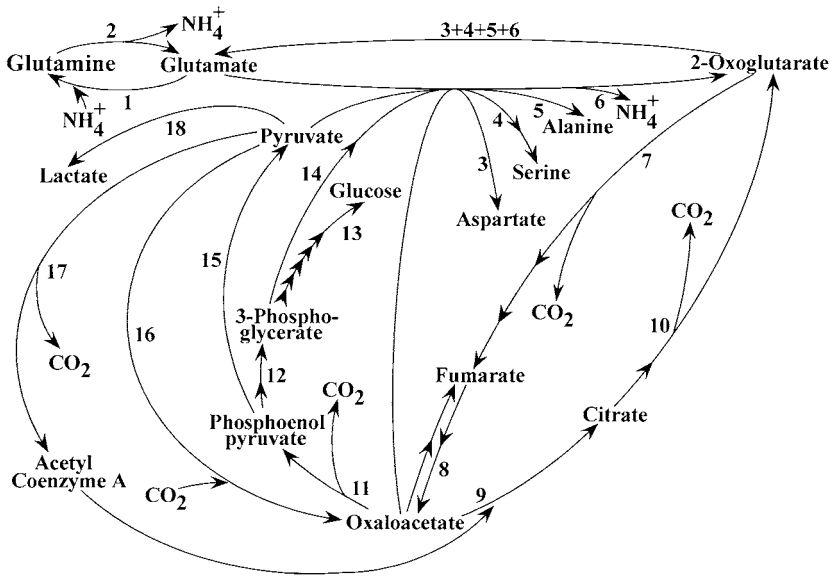
Net substrate utilization and product formation were calculated as the difference between the total flask contents (tissue plus medium) at the start (zero-time flasks) and after the period of incubation. The net metabolic rates, reported as means ± S.E.M., are expressed in μmol of substance removed or produced per unit time (30 or 60 min) per g of tubule dry weight.

The rates of release of $^{14}CO_2$ from the ^{14}C-labelled glutamine species used were calculated by dividing the radioactivity in $^{14}CO_2$ by the specific radioactivity of each labelled carbon of the glutamine species of interest measured in each medium.

Fixation of $^{13}CO_2$ from [^{13}C]bicarbonate was calculated by dividing the amount of acid-stable ^{13}C present in the incubation medium after incubation by the specific enrichment of the total 'bicarbonate + CO_2' pool measured in the zero-time flasks, which was 54 %.

When [^{13}C]glutamine species were the substrate, the transfer of the C-1, C-2 or C-3 of glutamine to a given position in a given metabolite was calculated by using the following formula: $(L_m - l_m)/(E_s - e_s)$, where L_m is the amount of ^{13}C measured in the corresponding NMR resonance, l_m is the amount of metabolite assayed enzymically multiplied by the natural ^{13}C abundance (1.1 %), E_s is the ^{13}C abundance of the C-1, C-2 or C-3 of glutamine, and e_s is the natural ^{13}C abundance. Note that $(E_s - e_s)$ is the specific enrichment of the corresponding species.

For interpretation of the data obtained, we used a mathematical model published previously [38] that is based on the incorporation of ^{13}C into various metabolites and allows the calculation of reaction rates of glutamine and glutamate degradation and synthesis, the TCA cycle (tricarboxylic acid cycle), gluconeogenesis, various transaminases as well as pyruvate kinase, pyruvate dehydrogenase and pyruvate carboxylase. Indeed, the two mathematical models available for renal [^{14}C]glutamine [32] and [^{13}C]glutamine [30] metabolism were not applicable to our data; the first one [32] is based on the assumption (which is not valid in our study) that all of the acetyl-CoA entering the TCA cycle is derived from fatty acids and not from glutamine, and the second one [30] does not involve all the enzymes responsible for glutamine metabolism in our study. In addition, these two models do not take into account the resynthesis of glutamate and glutamine or the operation of pyruvate carboxylase. Similarly, the sophisticated mathematical model developed by Jucker et al. [49] for studying



Scheme 1 Pathways of glutamine metabolism in the rat kidney

Numbers refer to enzymes involved in the different reactions: 1, glutamine synthetase; 2, glutaminase; 3, aspartate aminotransferase; 4, phosphoserine aminotransferase; 5, alanine aminotransferase; 6, glutamate dehydrogenase; 7, 2-oxoglutarate dehydrogenase; 8, malate dehydrogenase; 9, citrate synthase; 10, isocitrate dehydrogenase; 11, phosphoenolpyruvate carboxykinase; 12, phosphoglyceromutase; 13, glucose-6-phosphatase; 14, 3-phosphoglycerate dehydrogenase; 15, pyruvate kinase; 16, pyruvate carboxylase; 17, pyruvate dehydrogenase; 18, lactate dehydrogenase.

the hepatic metabolism of [^{13}C]ethanol in the rat, which allowed those authors to study glutamine and glutamate turnover, does not involve gluconeogenesis, a major process in our study.

The model of glutamine metabolism used in the present study is based on principles that have been used and validated in previous work from this laboratory for studying glucose, glutamate, alanine and acetate metabolism in isolated rabbit kidney tubules [34–37]. It is applicable under conditions where the accumulation and labelling of products of glutamine metabolism are approximately linear with time. This model, which combines proportions of metabolite conversion with substrate utilization, allows the calculation of fluxes related to glutamine metabolism.

The results obtained with tubules from fasted rats were compared with those obtained with tubules from fed rats by the unpaired two-tailed Student's t test; $n = 4$ was used in this statistical analysis.

RESULTS

In order to determine the precise pathways of glutamine carbon metabolism (see Scheme 1) and the effects of fasting on these pathways, we carried out two series of experiments in which we combined enzymic, radioactive and ^{13}C NMR spectroscopy measurements; for this, tubules from both fed and fasted rats were incubated for 60 min with specifically labelled [^{14}C]- or [^{13}C]glutamine. Preliminary experiments in which tubules from both fed and fasted rats were incubated for 30 and 60 min revealed that glutamine utilization and product formation and labelling were linear with time ($n = 2$ experiments with four kidneys from two rats in each experiment and in each nutritional state). In tubules from fed rats incubated for 30 and 60 min, the removal of glutamine and the accumulation of glutamate and glucose were 315 ± 25 and 603 ± 31 (30 and 60 min respectively), 100 ± 6 and 182 ± 11, and 46 ± 8 and 81 ± 3 μmol $\cdot$ h^{-1} $\cdot$ g dry wt^{-1} respectively; in tubules from fasted rats, the corresponding values were 322 ± 6 and 650 ± 7, 92 ± 13 and 176 ± 30, and 74 ± 4 and 140 ± 5 μmol $\cdot$ h^{-1} $\cdot$ g dry wt^{-1} respectively.

Table 1 Effects of fasting on the metabolism of 5 mM L-glutamine in rat kidney tubules

Kidney tubules (18.5 ± 0.4 and 16.3 ± 0.3 mg dry wt per flask for fed and fasted rats respectively) were incubated for 60 min as described in the Experimental section. Results for metabolite removal (−) or production are reported as means ± S.E.M. for four experiments performed in quadruplicate. The unpaired Student's t test was used to measure the statistical difference between tubules from fed and fasted rats: $^*P < 0.05$. The radioactivity and ^{13}C NMR data corresponding to these experiments are reported in Tables 2–4.

	Metabolite removal or production (μmol $\cdot$ h^{-1} $\cdot$ g dry wt^{-1})				
Nutritional state	Glutamine	Glutamate	Glucose	Alanine	Aspartate
Fed	-612 ± 17	164 ± 5	75 ± 2	9 ± 1	12 ± 1
Fasted	$-723 \pm 29^*$	175 ± 19	$125 \pm 6^*$	7 ± 1	$8 \pm 1^*$

Table 1 shows that, after 60 min of incubation, rat kidney tubules used glutamine (5 mM) as substrate at high rates, and that fasting caused a slight but statistically significant increase in substrate utilization. Despite the increase in glutamine utilization by tubules from fasted rats, glutamate accumulation remained unchanged when compared with that in tubules from fed animals. Most of the glutamate formed by glutaminase, the enzyme that initiates glutamine degradation, was metabolized further by glutamate dehydrogenase, as only small amounts of alanine and aspartate formed by alanine and aspartate aminotransferases were found to accumulate. Aspartate accumulation was decreased in tubules from fasted rats when compared with that in tubules from fed rats. Table 1 also shows that fasting caused a large increase in glucose synthesis from glutamine. Under no conditions did we observe any substantial accumulation of glycogen, ketone bodies, acetate, pyruvate, lactate or TCA cycle intermediates (citrate, 2-oxoglutarate, malate or fumarate). When incubated for 60 min

Table 2 Effects of fasting on the release of $^{14}CO_2$ from [1-^{14}C]- and [1,5-^{14}C]glutamine and on the accumulation of [^{13}C]glutamate, [^{13}C]glutamine, [^{13}C]serine and [^{13}C]glucose following the incorporation of $^{13}CO_2$ during glutamine metabolism in rat kidney tubules

Kidney tubules (18.5 ± 0.4 and 16.3 ± 0.3 mg dry wt per flask for fed and fasted rats respectively) were incubated for 60 min as described in the Experimental section. Results are reported as means ± S.E.M. for four experiments performed in quadruplicate. Substrate utilization and product formation measured enzymically are reported in Table 1. Statistical differences between tubules from fed and fasted rats were measured using the unpaired Student's *t* test: $^*P < 0.05$. $^{14}CO_2$ from [5-^{14}C]Gln was calculated as the difference between $^{14}CO_2$ from [1,5-^{14}C]Gln and $^{14}CO_2$ from [1-^{14}C]Gln.

	Release of $^{14}CO_2$ (μmol · h^{-1} · g dry wt^{-1}) from:			Accumulation from Gln + HCO_3^- (μmol · h^{-1} · g dry wt^{-1})				
Nutritional state	[1-^{14}C]Gln	[1,5-^{14}C]Gln	[5-^{14}C]Gln	[1-^{13}C]Gln	[1-^{13}C]Glu	[3-^{13}C]Glc	[4-^{13}C]Glc	[1-^{13}C]Ser
Fed	463 ± 8	832 ± 50	369 ± 44	11.3 ± 1.2	15.4 ± 2.5	5.9 ± 0.1	8.0 ± 0.5	10.8 ± 0.6
Fasted	548 ± 16*	935 ± 25	387 ± 25	11.4 ± 2.6	20.0 ± 2.0	12.0 ± 2.0*	15.7 ± 1.7*	8.1 ± 0.8*

Table 3 Effects of fasting on the metabolism of 5 mM [1,2-^{13}C]glutamine in rat kidney tubules

Kidney tubules (18.5 ± 0.4 and 16.3 ± 0.3 mg dry wt per flask for fed and fasted rats respectively) were incubated for 60 min as described in the Experimental section. C-1,2 denotes glutamine or glutamate molecules doubly labelled on C-1 and C-2. Results for ^{13}C-labelled metabolites removed (−) or accumulated are reported as means ± S.E.M. for four experiments performed in quadruplicate. Substrate utilization and product formation measured enzymically are reported in Table 1. The unpaired Student's *t* test was used to measure the statistical difference between tubules from fed and fasted rats: $^*P < 0.05$.

	Amount of labelled metabolites (μmol · h^{-1} · g dry wt^{-1})									
	Glutamine		Glutamate		Glucose		Aspartate		Lactate	Serine
Nutritional state	C-1,2	C-1	C-1,2	C-1	C-3	C-4	C-1	C-4	C-1	C-1
Fed	− 617 ± 12	12.8 ± 1.9	135 ± 5	10.9 ± 0.3	20.7 ± 1.1	23.8 ± 1.3	3.7 ± 2.1	2.5 ± 2.0	0.3 ± 0.2	8.7 ± 0.5
Fasted	− 730 ± 20*	7.6 ± 0.9*	129 ± 11	12.1 ± 1.4	36.2 ± 2.4*	38.9 ± 3.0*	4.7 ± 2.3	3.1 ± 2.0	2.9 ± 2.0	10.9 ± 2.3

in the absence of glutamine, glucose synthesis by tubules from fed and fasted rats was 6 ± 2 and 20 ± 5 μmol · h^{-1} · g dry wt^{-1} ($P < 0.05$).

The release of $^{14}CO_2$ from glutamine specifically labelled with ^{14}C is shown in Table 2. It can be seen that a large fraction of C-1 of glutamine was released as $^{14}CO_2$ by the 2-oxoglutarate dehydrogenase reaction, and that the C-1 released as CO_2 plus the glutamate accumulated (see Table 1) was approximately equal to the glutamine utilized in both nutritional states. Since very little alanine and aspartate accumulated, this means that most of the 2-oxoglutarate decarboxylated was formed by the glutamate dehydrogenase reaction. The difference between the release of $^{14}CO_2$ from [1,5-^{14}C]glutamine and that from [1-^{14}C]glutamine gives the release of $^{14}CO_2$ from C-5 of glutamine, which was 369 ± 44 and 387 ± 25 μmol · h^{-1} · g dry wt^{-1} in tubules from fed and fasted rats respectively (difference not significant). Since the amounts of C-2 and C-5 released as CO_2, which occurs beyond the symmetrical molecules succinate and fumarate, are assumed to be equal, one can deduce that the mean release of $^{14}CO_2$ from [2-^{14}C]glutamine was 369 ± 44 and 387 ± 25 μmol · h^{-1} · g dry wt^{-1} in tubules from fed and fasted rats respectively. Fasting increased the release as CO_2 of the C-1 of glutamine without any increase in glutamate accumulation, and substantial amounts of 2-oxoglutarate did not accumulate in tubules from either fed or fasted rats; therefore fasting increased flux through 2-oxoglutarate dehydrogenase during the first turn of the TCA cycle. In contrast, fasting did not statistically change the release of $^{14}CO_2$ from [1,5-^{14}C]glutamine; as shown in Table 2, the calculated release of CO_2 from C-5, and therefore from C-2, of glutamine remained statistically unaltered by fasting.

Table 2 also shows that, on incubation of tubules with unlabelled glutamine plus ^{13}C-labelled bicarbonate, fixation of $^{13}CO_2$ occurred and led to the labelling of C-1 of serine, glutamate and glutamine, and of C-3 and C-4 of glucose (NMR spectra not shown). This means that (i) pyruvate carboxylase operated during glutamine metabolism; (ii) part of the oxaloacetate synthesized by the latter reaction underwent equilibration with fumarate, as revealed by the labelling of C-1 of serine and C-3 and C-4 of glucose; thus the conversion 'oxaloacetate → malate/fumarate → oxaloacetate' also occurred thanks to the near-equilibrium reactions catalysed by malate dehydrogenase and fumarase that functioned in the backward and forward directions; and (iii) despite the utilization of glutamine and of part of the glutamine-derived glutamate, concomitant synthesis of glutamate and glutamine occurred, as indicated by the labelling of C-1 of glutamate and glutamine. Note that fasting did not alter the total label incorporated into the products found; similarly, it did not change the labelling of C-1 of glutamate and glutamine, but increased the labelling of C-3 and C-4 of glucose.

From the ^{13}C NMR spectra of $HClO_4$ extracts obtained after a 60 min incubation of kidney tubules from fed and fasted rats in the presence of [1,2-^{13}C]glutamine as substrate, we calculated the amounts of labelled products after correction for the natural abundance of ^{13}C, as described in the Experimental section. Table 3 shows that the uptake of [1,2-^{13}C]glutamine was very similar to the glutamine uptake measured enzymically (see Table 1). As expected, significant accumulation of [1,2-^{13}C]glutamate occurred; however, the accumulation of this labelled glutamate was significantly lower than that measured enzymically, which suggests that synthesis of glutamate unlabelled on C-1 and C-2 occurred mainly from the unlabelled carbons of the [1,2-^{13}C] glutamine added initially as substrate. Such a synthesis is in agreement with the synthesis of glutamate observed above in the presence of glutamine plus [^{13}C]bicarbonate (see Table 2). As C-1 of glutamine is released as CO_2 by 2-oxoglutarate dehydrogenase, it is obvious that only the labelled C-2 of this substrate could lead to labelling of products beyond 2-oxoglutarate. Table 3 shows that C-1 of aspartate, lactate and serine, C-4 of aspartate and C-3 and C-4 of glucose were labelled. This is in agreement with the symmetrical nature of succinate and fumarate, which led

to the labelling of the C-1 and the C-4 of oxaloacetate. Another important finding is that singlets of C-1 of glutamate and glutamine were found to accumulate. This indicates that C-1 of the latter compounds became labelled from C-2 of glutamine, and therefore that concomitant degradation and synthesis of glutamate and glutamine occurred. In agreement with results already observed with enzymic measurements (Table 1), Table 3 shows that fasting caused a statistically significant stimulation of glutamine utilization and glucose labelling, a diminution of the labelling of C-1 of glutamine, but no change in the accumulation of labelled glutamate.

Table 3 shows that, out of 463 μmol $\cdot$ h^{-1} $\cdot$ g dry wt^{-1} of oxaloacetate synthesized (flux through 2-oxoglutarate dehydrogenase during the first TCA cycle turn) in tubules from fed rats, 89 μmol $\cdot$ h^{-1} $\cdot$ g dry wt^{-1} of oxaloacetate [twice the amount of C-3 and C-4 of glucose (44.5 μmol $\cdot$ h^{-1} $\cdot$ g dry wt^{-1}) labelled from [2-^{13}C]glutamine] was converted into glucose; therefore, neglecting the small amounts of aspartate, serine, alanine and lactate found to accumulate, most of the remainder of the oxaloacetate formed was metabolized further either through citrate synthase or through pyruvate dehydrogenase after prior conversion into pyruvate by the phosphoenolpyruvate carboxykinase and the pyruvate kinase reactions. Thus only a small fraction (89/463 = 19 %) of the oxaloacetate synthesized during the first and subsequent turns of the TCA cycle was directed to glucose. Therefore several TCA cycle turns were needed to synthesize the glucose found enzymically, because the latter was severalfold greater than the amounts of [3-^{13}C]- and [4-^{13}C]glucose found. Similar calculations from the data presented in Table 3 reveal that, in tubules from fasted rats, 27 % (150/548) of the oxaloacetate molecules synthesized during the first and subsequent turns of the TCA cycle were directed to glucose; again this means that several TCA cycle turns were needed to synthesize glucose whose production and labelling was greatly stimulated by fasting (see Tables 1 and 3).

The ^{13}C NMR spectra obtained after a 60 min incubation of tubules from fed and fasted rats in the presence of [3-^{13}C] glutamine as substrate are shown in Figure 1. The corresponding values are presented in Table 4. Again, the amount of [3-^{13}C] glutamine utilized was in good agreement with the glutamine utilizations measured both enzymically and with [1,2-^{13}C]glutamine as substrate (see Tables 1 and 3). The amount of [3-^{13}C] glutamate that accumulated tended to be higher than the [1,2-^{13}C]glutamate accumulated from [1,2-^{13}C]glutamine, as a result of the possibility for the tubules to resynthesize glutamate labelled on C-3 from [3-^{13}C]glutamine, but not glutamate labelled on C-1 and C-2 from [1,2-^{13}C]glutamine, after the first TCA cycle turn. As expected, the main non-volatile product of [3-^{13}C]glutamine metabolism was glucose, the carbon atoms of which were all found to be labelled. The fact that C-1, C-2, C-5 and C-6 of glucose were found to be labelled to the same extent is due to the symmetrical nature of glucose synthesis which occurred from either [2-^{13}C]oxaloacetate or [3-^{13}C]oxaloacetate after the passage of C-3 of [3-^{13}C]glutamine through the symmetrical molecules succinate and fumarate. The fact that C-3 and C-4 of glucose were also labelled clearly indicates that part of the oxaloacetate formed from glutamine was converted into citrate by citrate synthase to give rise, after a complete TCA cycle turn, to [1-^{13}C]oxaloacetate and [4-^{13}C]oxaloacetate that were directed to glucose, with the C-4 of oxaloacetate being released as CO_2 by the phosphoenolpyruvate carboxykinase reaction.

Out of the 117 μmol $\cdot$ h^{-1} $\cdot$ g dry wt^{-1} of C-1 + C-2 + C-5 + C-6 of glucose found to be labelled from [3-^{13}C]glutamine in tubules from fed rats, 89 μmol $\cdot$ h^{-1} $\cdot$ g dry wt^{-1} (or 76 %) resulted from the conversion of the oxaloacetate synthesized during the first turn of the TCA cycle (i.e. just after the conversion of glutamine into oxaloacetate; see the results in Tables 3 and 4) and the remainder was labelled after additional TCA cycle turns. Similar calculations indicate that, in tubules from fasted rats, 150 out of 177 μmol $\cdot$ h^{-1} $\cdot$ g dry wt^{-1} (or 85 %) of the labelled C-1 + C-2 + C-5 + C-6 of glucose resulted from the conversion of the C-3 of [3-^{13}C]glutamine into glucose carbons during the first TCA cycle turn. In agreement with the increased glucose synthesis measured enzymically (Table 1) and with the increased conversion of C-2 of [1,2-^{13}C]glutamine into glucose (Table 3), the results given in Table 4 demonstrate that more C-3 of glutamine was converted into glucose in tubules from fasted rats than in those from fed rats.

It can also be seen from the data given in Table 4 that C-1, C-2, C-4 and C-5 of glutamate and glutamine, which were unlabelled at the start of the incubation period, became labelled during incubation with [3-^{13}C]glutamine. This confirms the existence of concomitant degradation and synthesis of both glutamate and glutamine, with these processes contributing to the turnover of these amino acids. Moreover, the labelling of C-1 and C-2 (which also indicates a resynthesis of C-3) of glutamate and glutamine fits with the view that a substantial fraction of the oxaloacetate synthesized during the first turn of the TCA cycle was recycled (see above). The fact that C-3 of glutamine was converted into C-4 and C-5 of glutamate and glutamine indicates that part of the oxaloacetate formed from glutamine was metabolized through phosphoenolpyruvate carboxykinase, pyruvate kinase, pyruvate dehydrogenase and then citrate synthase. The labelling of C-2 and C-3 of lactate is in agreement with the synthesis of pyruvate from glutamine.

The observation that, in tubules from both fed and fasted rats, C-2 of glutamate and glutamine contained more label than C-1 of these molecules reflects the fact that C-2 (and C-3) of glutamate and glutamine, derived from C-3 (and C-2) of oxaloacetate, can be labelled to a substantial extent during the second turn of the TCA cycle, whereas C-1 of glutamate and glutamine, derived from C-4 of oxaloacetate, cannot be labelled before the third TCA cycle turn. Another important piece of information provided by the data of Table 4 is that C-2 of glutamate and glutamine contained more label than C-4 or C-5 of these molecules. This reflects the fact that more oxaloacetate was immediately converted into citrate by citrate synthase than into acetyl-CoA (whose carbons give rise to C-4 and C-5 of glutamate and glutamine) by phosphoenolpyruvate carboxykinase, pyruvate kinase and pyruvate dehydrogenase.

Note that, in the presence of [3-^{13}C]glutamine as substrate, the sum of the carbons labelled in glutamate and glutamine synthesized, which is equal to the sum of the labelled C-1, C-4 and C-5 and twice the sum of the labelled C-2 (because C-2 and the C-3 of glutamate and glutamine are necessarily equally labelled from the labelled C-3 and C-2 of oxaloacetate respectively), was significantly diminished in tubules from fasted when compared with that in tubules from fed rats (50 compared with 108 μmol $\cdot$ h^{-1} $\cdot$ g dry wt^{-1}). The data of Table 4 show that this was due to a decrease in the labelling of the carbons of both glutamate and glutamine. Finally, Table 4 shows that the labelling of alanine, aspartate, serine and lactate was unaffected by fasting.

The observation that the amount of ^{13}C-labelled aspartate and alanine found (Table 4) exceeded the formation of alanine and aspartate measured enzymically (Table 1) can be explained by a small utilization of the aspartate and alanine present in the flasks at time zero and their replacement by ^{13}C-labelled alanine and aspartate.

The sum of the C-3 of [3-^{13}C]glutamine found in glucose, glutamate, glutamine, serine, alanine and aspartate and lactate was almost the same in tubules from fed and fasted rats (398 and

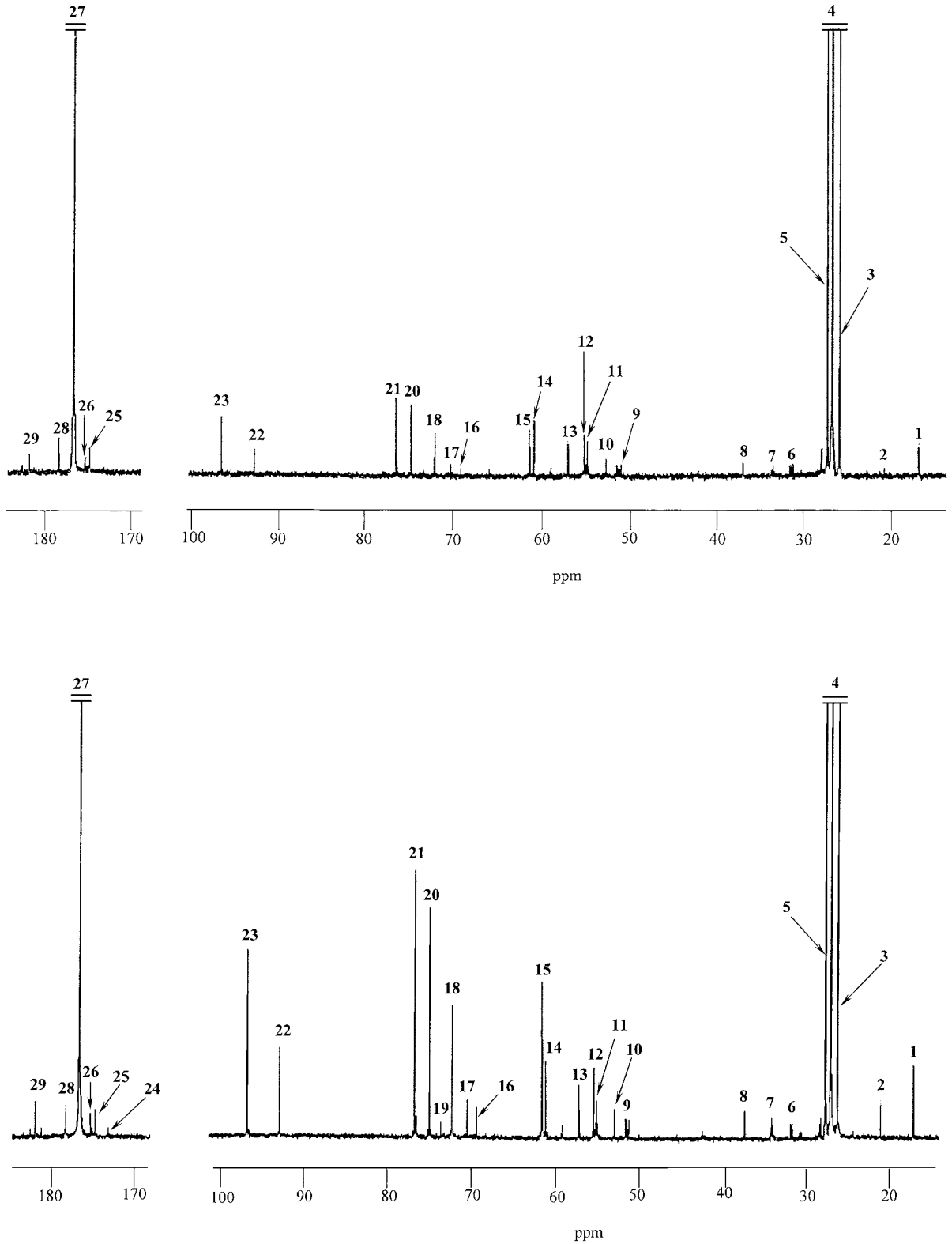


Figure 1 **^{13}C NMR spectra (125.75 MHz) of neutralized $HClO_4$ extracts obtained from fed (upper panel) and fasted (lower panel) rat kidney tubules incubated with [3-^{13}C]glutamine**

Peaks are identified as follows (values are p.p.m.): 1, alanine C-3 (17.01); 2, lactate C-3 (20.97); 3, contaminant (26.08); 4, glutamine C-3 (26.90); 5, glutamate C-3 (28.1); 6, glutamine C-4 (31.61); 7, glutamate C-4 (33.95); 8, aspartate C-3 (37.21); 9, alanine C-2 (51.27); 10, aspartate C-2 (52.76); 11, glutamine C-2 (54.88); 12, glutamate C-2 (55.25); 13, serine C-2 (57.02); 14, serine C-3 (60.90); 15, α,β-glucose C-6 (61.3); 16, lactate C-2 (69.12); 17, α,β-glucose C-4 (70.28); 18, α-glucose C-2,5 (72.05); 19, α-glucose C-3 (73.40); 20, β-glucose C-2 (74.79); 21, β-glucose C-3,5 (76.50); 22, α-glucose C-1 (92.74); 23, β-glucose C-1 (96.49); 24, serine C-1 (173.31); 25, glutamine C-1 (174.89); 26, glutamate C-1 (175.38); 27, alanine C-1 (176.7); 28, glutamine C-5 (178.29); 29, glutamate C-5 (181.96). The chemical shifts in p.p.m. are referred to tetramethylsilane.

Table 4 Effects of fasting on the metabolism of 5 mM [3-^{13}C]glutamine in rat kidney tubules

Kidney tubules (18.5 ± 0.4 and 16.3 ± 0.3 mg dry wt per flask for fed and fasted rats respectively) were incubated for 60 min as described in the Experimental section. Results for ^{13}C-labelled metabolite removed (−) or accumulated are reported as means ± S.E.M. for four experiments performed in quadruplicate. Substrate utilization and product formation measured enzymically are reported in Table 1. The unpaired Student's *t* test was used to measure the statistical difference between tubules from fed and fasted rats: $^*P < 0.05$.

	Amount of labelled metabolites (μmol · h^{-1} · g dry wt^{-1})											
	Glutamine					Glutamate					Aspartate	
Nutritional state	C-1	C-2	C-3	C-4	C-5	C-1	C-2	C-3	C-4	C-5	C-2	C-3
Fed	6.4 ± 2.1	11.9 ± 1.8	− 592 ± 18	3.2 ± 0.2	4.5 ± 0.4	12.1 ± 1.4	23.1 ± 2.1	145 ± 8	5.5 ± 0.4	6.7 ± 0.4	7.1 ± 0.9	6.8 ± 0.9
Fasted	1.2 ± 0.5*	6.2 ± 1.0*	− 694 ± 15*	1.0 ± 0.2*	1.8 ± 0.2*	4.3 ± 1.4*	11.3 ± 0.8*	166 ± 20	3.5 ± 0.9	3.1 ± 0.7*	5.5 ± 0.3	6.3 ± 0.7

	Amount of labelled metabolites (μmol · h^{-1} · g dry wt^{-1})												
	Lactate		Alanine		Serine			Glucose					
Nutritional state	C-2	C-3	C-2	C-3	C-1	C-2	C-3	C-1	C-2	C-3	C-4	C-5	C-6
Fed	1.5 ± 0.6	1.8 ± 0.6	6.2 ± 0.5	4.4 ± 0.4	1.9 ± 0.7	9.5 ± 0.5	11.5 ± 0.4	30.4 ± 3.4	27.6 ± 2.9	6.3 ± 0.9	6.0 ± 0.9	28.9 ± 3.0	30.4 ± 2.8
Fasted	4.1 ± 2.0	5.3 ± 0.4*	4.8 ± 1.3	5.1 ± 0.6	1.0 ± 0.6	8.9 ± 0.5	8.9 ± 0.6	46.1 ± 4.5*	44.6 ± 3.2*	4.6 ± 0.7	5.7 ± 0.9	40.9 ± 2.9*	45.4 ± 4.1*

436 μmol · h^{-1} · g dry wt^{-1} respectively). The difference between flux through 2-oxoglutarate dehydrogenase during the first turn of the TCA cycle (given by the release of $^{14}CO_2$ from [1-^{14}C] glutamine) and the sum of the labelled carbons found in the latter products gives an estimate of the C-3 of glutamine presumably released as CO_2, which was equal to 174 ± 32 and 261 ± 22 μmol · h^{-1} · g dry wt^{-1} in tubules from fed and fasted rats respectively. As C-3 and C-4 of glutamine behave symmetrically after having been converted into succinate by the successive action of glutaminase, glutamate dehydrogenase or serine or alanine or aspartate aminotransferases, 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase, the latter values also apply to the conversion into CO_2 of C-4 of glutamine in tubules from fed and fasted rats.

Thus, in tubules from fed rats, the mean values for the conversion of C-1, C-2, C-3, C-4 and C-5 of glutamine into CO_2 are 463, 369, 174, 174 and 369 μmol · h^{-1} · g dry wt^{-1} respectively; the corresponding values in tubules from fasted rats are 548, 387, 261, 261 and 387 μmol · h^{-1} · g dry wt^{-1} respectively. Complete oxidation of the glutamine carbon skeleton, i.e. oxidation in which all carbons are converted into CO_2, is defined by the smallest common denominator, which is the conversion of C-3 or C-4 of glutamine into CO_2. Therefore the mean complete oxidation of glutamine in tubules from fed and fasted rats was 174 ± 32 and 261 ± 22 μmol · h^{-1} · g dry wt^{-1} respectively (not significantly different). This suggests that complete oxidation of glutamine in rat kidney tubules, which involves the mitochondrial exit of malate and the combined action of phosphoenolpyruvate carboxykinase (the activity of which is more than 98 % cytosolic [50]), pyruvate kinase, pyruvate dehydrogenase and the TCA cycle, was not changed by fasting, although the *P* value (0.07) was close to statistical significance. Although the observed change is not statistically significant, this follows the same trend as the change in glutamine removal and CO_2 production from C-1 of glutamine. The lack of significance was probably due to the scatter in the data that were not directly measured but rather calculated.

It is also possible to calculate the partial oxidation of the glutamine carbon skeleton as the difference between flux through 2-oxoglutarate dehydrogenase during the first turn of the TCA cycle and the complete oxidation of glutamine; thus, in tubules from fed and fasted rats, the estimates of the mean partial oxidation of glutamine were virtually identical (289 ± 25 and 287 ± 18 μmol · h^{-1} · g dry wt^{-1} respectively). Overall, these results underline the importance of glutamine carbon oxidation and the fact that the individual glutamine carbons contribute to a different degree to the production of CO_2. They also show that, during fasting, only C-1 of glutamine increased substantially its contribution to the production of CO_2.

Table 5 shows the absolute values of fluxes through the enzymes involved in glutamine metabolism in tubules from fed and fasted rats. These values are derived from the combination of proportions that constitute the basis of our model [38] with substrate utilization and in particular ^{13}C NMR spectroscopy data. As expected, glutamine degradation by glutaminase occurred at high rates, and glutamine synthesis also occurred at substantial rates. Fasting was found to stimulate flux through glutaminase, but did not alter flux through glutamine synthetase. Net glutamine utilization, calculated as flux through glutaminase minus flux through glutamine synthetase, was similar to glutamine utilization measured enzymically (see Table 1 for comparison). Table 5 also shows that net flux through glutamate dehydrogenase, but not through alanine, aspartate and phosphoserine aminotransferases, was stimulated by fasting; the increase in net flux through glutamate dehydrogenase in the oxidative deamination direction resulted from a diminution of the unidirectional flux through this enzyme in the direction of reductive amination (Table 5).

In agreement with the recycling of oxaloacetate through the TCA cycle mentioned above, total flux through 2-oxoglutarate dehydrogenase, which was not statistically changed by fasting, was approx. 3-fold higher than flux through the latter enzyme during the first turn of the TCA cycle (see the release of $^{14}CO_2$ from [1-^{14}C]glutamine in Table 2). As can be seen in Table 5, fasting substantially stimulated not only fluxes through phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, but also fluxes through pyruvate kinase and pyruvate carboxylase. In contrast, fluxes through pyruvate dehydrogenase and citrate synthase remained unaffected by fasting.

DISCUSSION

To our knowledge, the present work is the first in which the complexity of renal glutamine metabolism in the rat has been studied thoroughly. Although a substantial number of studies have been devoted to renal glutamine metabolism in the rat (see for

Table 5 Effects of fasting on fluxes through pathways of glutamine metabolism in rat kidney tubules

Values, reported as means ± S.E.M. for four experiments performed in quadruplicate, were calculated from Tables 1–4 as explained in the text. The unpaired Student's *t* test was used to measure the statistical differences between tubules from fed and fasted rats: $*P < 0.05$. Abbreviations: GS, glutamine synthetase; 2OG, 2-oxoglutarate; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; PSerAT, phosphoserine aminotransferase; ODH, 2-oxoglutarate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; LDH, lactate dehydrogenase; CS, citrate synthase; G6Pase, glucose-6-phosphatase.

	Flux (μmol of C_3 units · h^{-1} · g dry wt^{-1})							
			Glutamate dehydrogenase					
Nutritional state	Glutaminase	GS	{Glu → 2OG}	{2OG → Glu}	Net {Glu → 2OG}	AlaAT	AspAT	PSerAT
Fed	804 ± 22	199 ± 27	516 ± 23	143 ± 8	374 ± 20	13 ± 1	15 ± 1	21 ± 1
Fasted	945 ± 47*	229 ± 34	547 ± 17	78 ± 9*	469 ± 20*	12 ± 1	15 ± 2	18 ± 2

	Flux (μmol of C_3 units · h^{-1} · g dry wt^{-1})							
Nutritional state	ODH	PEPCK	PK	PC	PDH	LDH	CS	G6Pase
Fed	1446 ± 80	1028 ± 56	877 ± 57	588 ± 40	273 ± 26	4 ± 1	1006 ± 85	129 ± 3
Fasted	1619 ± 77	1362 ± 49*	1123 ± 48*	821 ± 51*	278 ± 36	12 ± 3	1085 ± 79	221 ± 14*

example [26–33]), none of them has identified and quantified not only the fate of each individual carbon atom of glutamine but also the metabolic pathways involved, as we have done in the present study. This was made possible by the concomitant use of enzymic, radioactive and ^{13}C NMR spectroscopy methods in combination with a model of glutamate and glutamine metabolism developed previously [38]. Note that, in contrast with the two models of glutamine metabolism described by Vinay et al. [32] and Nissim and Yudkoff [30], our model allowed us to calculate fluxes through all enzymes substantially involved in renal glutamine metabolism. Note also that, to our knowledge, ^{13}C NMR spectroscopy has so far been used to study glutamine metabolism in only one study in rat liver *in vivo* with [^{13}C]alcohol as substrate [49]. Nissim and Yudkoff [30] also studied the use of [3-^{13}C,5-^{15}N]glutamine as substrate in renal tubules from rats in different acid–base conditions, and utilized GC-MS to measure the appearance of ^{13}C in glucose and TCA cycle intermediates.

Pathways of glutamine metabolism

The experimental approach used in the present study reveals that the description of renal glutamine metabolism as a single passage from glutamine to the different end-products, mainly glutamate, glucose and CO_2 on the basis of measurements performed with enzymic methods, is simplistic. Labelling data and calculations of fluxes through various enzymes potentially involved in such metabolism reveal an unexpected degree of complexity of such metabolism. Irrespective of the nutritional state of the rats studied, renal glutamine utilization measured enzymically or as the removal of [^{13}C]glutamine was underestimated and represented only net glutamine utilization, because substantial synthesis of glutamine also occurred (see Table 5). Similarly, glutamate was not only degraded mainly by glutamate dehydrogenase, but also synthesized by the same enzyme. Note that these concomitant syntheses and degradations of glutamine and glutamate, which were demonstrated by the presence of glutamate and glutamine molecules labelled on carbon atoms different from that labelled initially in the added substrate, represent a particular kind of bidirectional flux and futile cycle in which the 2-oxoglutarate, glutamate and glutamine molecules synthesized were labelled on a carbon atom different from that in the added glutamine molecule. However, our data indicate that there were also true futile cycles involving glutamine, glutamate and 2-oxoglutarate molecules with the same labelling pattern that rendered their existence difficult to detect; the fact that the uptake of [1,2-^{13}C]glutamine was less than flux through glutaminase is in agreement with the existence of a true futile cycle between glutamine and glutamate.

An important piece of information is that recycling in the TCA cycle of the oxaloacetate synthesized during the first turn of TCA cycle also occurred, and contributed substantially not only to the resynthesis of glutamate and glutamine but also to gluconeogenesis. This is why the total flux through 2-oxoglutarate dehydrogenase was approx. 3-fold higher (see Table 5) than the flux through the same enzyme during the first turn of TCA cycle (see Table 2). The importance of such recycling of glutamine carbon atoms through the TCA cycle and the consequences for calculating gluconeogenesis from [U-^{14}C]glutamine have also been underlined in a previous study [32].

Although it is in theory not necessary for gluconeogenesis from glutamine, pyruvate carboxylase was highly involved in glutamine metabolism by rat kidney tubules; together with the equilibration of oxaloacetate with fumarate thanks to the reversible part of the TCA cycle between oxaloacetate and fumarate, it also participated in the resynthesis of both glutamate and glutamine (see Table 2). Thus four cycles and bidirectional fluxes through the near-equilibrium enzymes glutamate dehydrogenase, malate dehydrogenase and fumarase operated simultaneously during glutamine metabolism in our rat kidney tubules from fed and fasted rats: a 'glutamine → glutamate → glutamine' cycle, an 'oxaloacetate → phosphoenolpyruvate → pyruvate → oxaloacetate' cycle, the TCA cycle and the 'oxaloacetate → phosphoenolpyruvate → pyruvate → acetyl-CoA → citrate → oxaloacetate' cycle.

The fact that high fluxes through pyruvate kinase and pyruvate dehydrogenase occurred in our rat tubules is in agreement with the previous demonstration of high activities of these enzymes in kidney tubules from both fed and fasted rats [51].

Effects of fasting on fluxes through enzymes of glutamine metabolism

The stimulation of glutamine utilization by tubules from fasted rats in the present study is in agreement with the stimulation of glutamine removal by the kidney of fasted rats *in vivo* [25]. The conclusion that this was due to stimulation of flux through glutaminase, but not to a diminution of flux through glutamine synthetase or both, represents a new piece of information. At first

sight, the absence of an effect of fasting on flux through glutamine synthetase may appear surprising, because the synthesis of labelled glutamine carbons from [3-^{13}C]glutamine decreased dramatically in tubules from fasted rats when compared with tubules from fed rats (see Table 4). However, it should be underlined that the amount of labelled carbons re-incorporated into glutamine represents only a fraction of the total glutamine resynthesized. In fact, the decreased re-incorporation of ^{13}C into glutamine indicates that the specific enrichment of the glutamate pool was decreased by fasting; this is in agreement with the decreased unidirectional flux of glutamate dehydrogenase in the direction of reductive amination (Table 5).

The observation that net flux through glutamate dehydrogenase in the direction of glutamate deamination was stimulated by fasting is in agreement with the absence of increases in glutamate accumulation (see Tables 1, 3 and 4) and in fluxes through alanine, aspartate and phosphoserine aminotransferases, despite increased glutamate synthesis by glutaminase. The stimulation of glutamine removal and of net glutamate deamination in tubules from fasted rats is consistent with the view emphasized by Nissim [52], and Nissim and co-workers [53] that stimulation of glutamate removal should occur to stimulate flux through glutaminase because of the inhibitory effect of glutamate on the latter enzyme.

In agreement with the well-established stimulation by fasting of phosphoenolpyruvate carboxykinase activity in the rat kidney cortex [42,43,54,55] as a result of increased gene expression (for a review see [56]), flux through the latter enzyme was stimulated in tubules from fasted rats. This stimulation, which led not only to the stimulation of flux through the subsequent steps of gluconeogenesis but also to the stimulation of flux through pyruvate kinase, was also probably responsible for the decreased accumulation of aspartate found in tubules from fasted rats (see Table 1). The resulting increase in pyruvate synthesis was not accompanied by stimulation of pyruvate utilization by pyruvate dehydrogenase, lactate dehydrogenase or alanine aminotransferase, but rather by stimulation of flux through pyruvate carboxylase. It is interesting to note that, in agreement with our observations reported in Table 5 that fluxes through pyruvate carboxylase and glucose-6-phosphatase were augmented in tubules from fasted rats, fasting has also been shown to stimulate the activity of these enzymes in the rat kidney cortex [43,54]. Finally, fasting statistically altered neither flux through citrate synthase nor total flux through 2-oxoglutarate dehydrogenase, which suggests that the recycling of oxaloacetate through the TCA cycle did not change in rat kidney tubules as a result of this nutritional state.

Contribution of intra- and intercellular metabolism to the recycling of metabolites

Since glutamine synthetase is restricted to the straight portion of the rat proximal tubule [43,44], it is obvious that our tubule suspensions contained not only convoluted segments but also straight segments of proximal tubules. To our knowledge, the respective proportions of convoluted and straight segments contained in proximal tubule suspensions, and therefore their respective contributions to glutamine metabolism, have so far not been determined. However, knowledge of the distribution along the rat nephron of the enzymes involved in glutamine metabolism (for a review see [57]) together with the characteristics of this metabolism observed in the present study allow us to discuss this question. Although phosphate-dependent glutaminase is present along the entire rat proximal tubule, its activity is much lower in the straight segment than in the convoluted segment [23,58]; however, calculations indicate that, even in the straight segment, its capacity [23,58] exceeds the flux through glutaminase found in our present study. Thus glutamine may well be utilized by the terminal portion of the proximal tubule. In agreement with this view is the demonstration that the straight portion of the rat proximal tubule produced substantial amounts of $^{14}CO_2$ from [U-^{14}C]glutamine [59]. Thus, taking into account the distribution along the rat nephron of the mitochondrial and cytosolic enzymes involved in glutamine degradation [57], it appears that all proximal tubular segments are able to metabolize glutamine carbon into glutamate, glucose and CO_2. Therefore, provided that these enzymes are present in the same cells (a fact that remains to be demonstrated), bidirectional fluxes through glutamate dehydrogenase, malate dehydrogenase and fumarase, and flux through the futile cycle involving pyruvate carboxylase, phosphoenolpyruvate carboxykinase and pyruvate kinase, as well as flux through the TCA cycle, could occur intracellularly in all of the proximal segments present in our tubule suspensions. In contrast, only segments derived from the pars recta, to which glutamine synthetase is restricted [43,44], would be able to synthesize glutamine. In this respect, it should be mentioned that Schoolwerth et al. [44] reported that glutamine synthetase on the one hand, and glutaminase and glutamate dehydrogenase proteins on the other hand, are localized in different cells in the rat pars recta. Accordingly, the concomitant degradation and synthesis of glutamine observed in our present study could occur only intercellularly. Therefore ^{13}C NMR spectroscopy allowed us to study the metabolism of different intermingled cell populations without separating them. It should also be underlined here that it is quite conceivable that, *in vivo*, like *in vitro*, intercellular co-operation with respect to glutamine degradation and synthesis can also occur between cells of the proximal convoluted tubule and those of the proximal straight tubule.

It is important to mention that we used in the present study a glutamine concentration (5 mM) that is much higher than the plasma levels of this amino acid (0.5–0.8 mM). We deliberately chose to use such a high glutamine concentration and a large amount of kidney tubules so that it was possible to measure in a reliable manner the rates of substrate removal and product formation, to compensate for the poor sensitivity of ^{13}C NMR spectroscopy. The fact that both gluconeogenesis and oxidation of glutamine result in a net production of ATP [6] raises the question of whether the high glutamine concentration used might have induced glutamate accumulation and ATP-wasting cycles such as the glutamine/glutamate cycle. In this respect, it should be underlined that the rate of glutamine utilization by rat kidney tubules is only approximately doubled when the glutamine concentration is increased from a near-physiological concentration (1 mM) [60] to 5 mM (present study). Carbon balance calculations also reveal that, at both 1 and 5 mM glutamine, a high rate of complete oxidation of the glutamine carbon skeleton removed occurs ([60]; present study); this suggests that the pathways of glutamine metabolism observed with 5 mM glutamine are also functional with more physiological concentrations of this amino acid. In agreement with this view is the observation that, despite low glutamate accumulation, concomitant degradation and synthesis of glutamine occurred when glutamine was used at a concentration of 1 mM [60]. Whether this also applies to bidirectional fluxes and to the oxaloacetate → phosphoenolpyruvate → pyruvate → oxaloacetate cycle observed in the present study remains to be verified.

Physiological significance

The fact that complete and partial oxidations of the glutamine carbon skeleton were not altered in tubules from fasted rats strongly suggests that, in the rat kidney *in vivo*, like in our tubules

in vitro, glutamine is an important energy provider for renal reabsorptive work irrespective of the nutritional state.

Although isotopic studies performed in the rat *in vivo* have suggested that renal gluconeogenesis contributes substantially to systemic gluconeogenesis [61], the physiological significance of glucose synthesis from glutamine and of its stimulation by fasting is difficult to evaluate; indeed, arteriovenous difference values for unlabelled or labelled glucose across the rat kidney are small and often represent only a few per cent of the arterial glucose concentration, and therefore are within the margin of error of the analytical methods used [25,61]. However, it should be underlined that the difficulty in measuring precisely renal gluconeogenesis *in vivo* does not mean that this process is not quantitatively important.

The existence of bidirectional fluxes through certain near-equilibrium enzymes and of several futile cycles in glutamine metabolism by rat kidney tubules may appear surprising, and raises the question of their existence and significance under *in vivo* conditions. In this respect, the glutamine → glutamate → glutamine cycle and the concomitant degradation and synthesis of glutamate have been demonstrated *in vivo* [24]; as in our tubules *in vitro*, they probably represent a sophisticated means for the renal cells to regulate fluxes through the three enzymes that control not only glutamine carbon availability for glucose production and energy provision but also the renal production of the ammonium ions required to defend the systemic acid–base equilibrium. Whether bidirectional fluxes through malate dehydrogenase and fumarase and the oxaloacetate → phosphoenolpyruvate → pyruvate → oxaloacetate cycle operate in the rat kidney *in vivo* remains to be demonstrated. However, it is very likely that the latter cycle operates *in vivo*, because the demonstrated synthesis of glutamate and glutamine from 2-oxoglutarate in the intact functioning rat kidney *in vivo* [24] implies that the resulting depletion of TCA cycle intermediates should be replenished by an anaplerotic reaction. Finally, the fact that the net utilization of glutamine was increased in tubules from fasted rats, due to the stimulation of fluxes through glutaminase and glutamate dehydrogenase, is in agreement with the stimulation of glutamine utilization and ammonium production observed *in vivo* in the kidneys of fasted rats [25].

Conclusions

By combining the metabolic balance of substrates, incorporation of label into metabolites of glutamine and a model of glutamate and glutamine metabolism, we clearly demonstrate that glutamine metabolism in isolated rat kidney tubules is much more complicated than previously thought. In particular, the futile cycle involving phosphoenolpyruvate carboxykinase, pyruvate kinase and the anaplerotic enzyme pyruvate carboxylase appears to be an essential and regulated component of this metabolism. Moreover, recycling of the glutamine-derived oxaloacetate through the TCA cycle was substantial in tubules from both fed and fasted rats. The results obtained also show that bidirectional flux occurred through glutamate dehydrogenase, and that glutamine synthetase operated concomitantly with glutaminase.

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