Glutamine gluconeogenesis in the small intestine of 72 h-fasted adult rats is undetectable

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Recent reports have indicated that 48–72 h of fasting, Type 1 diabetes and high-protein feeding induce gluconeogenesis in the small intestine of adult rats *in vivo*. Since this would (i) represent a dramatic revision of the prevailing view that only the liver and the kidneys are gluconeogenic and (ii) have major consequences in the metabolism, nutrition and diabetes fields, we have thoroughly re-examined this question in the situation reported to induce the highest rate of gluconeogenesis. For this, metabolically viable small intestinal segments from 72 h-fasted adult rats were incubated with [3-¹³C]glutamine as substrate. After incubation, substrate utilization and product accumulation were measured by enzymatic and NMR spectroscopic methods. Although the segments utilized [¹³C]glutamine at high rates and accumulated

¹³C-labelled products linearly for 30 min *in vitro*, no substantial glucose synthesis could be detected. This was not due to the re-utilization of [¹³C]glucose initially synthesized from [¹³C]glutamine. Arteriovenous metabolite concentration difference measurements across the portal vein-drained viscera of 72 h-fasted Wistar and Sprague–Dawley rats clearly indicated that glutamine, the main if not the only gluconeogenic precursor taken up, could not give rise to detectable glucose production *in vivo*. Therefore we challenge the view that the small intestine of the adult rat is a gluconeogenic organ.

Key words: gluconeogenesis, glucose synthesis, glutamine metabolism, ketone body, NMR spectroscopy, small intestine.

INTRODUCTION

The contribution of the liver to glucose production in normal fasted humans and animals is well established; under this situation, the liver produces glucose as a result of both gluconeogenesis mainly from lactate, alanine, glutamine and glycerol and glycogenolysis when glycogen stores are available [1,2]. Various studies performed in humans and, to a lesser extent, in rats have suggested that, besides the liver, the kidneys may contribute to systemic gluconeogenesis; indeed, it has been reported that renal gluconeogenesis may represent 5–45% of systemic gluconeogenesis in post-absorptive or fasted humans (see [3] for a recent review).

Combining isotopic techniques with or without arteriovenous concentration difference measurements or organ vessel ligation, Mithieux and co-workers [4–7] recently published a series of papers assessing the contribution of the small intestine of adult rats to systemic endogenous glucose production under various experimental conditions. In agreement with the findings of Watford et al. [8] and of Baverel and Lund [9] with isolated enterocytes from fed rats, they did not observe any intestinal gluconeogenesis *in vivo* in control rats fasted for 5–6 or 24 h [4,5], although they more recently found the reverse in 24 h-fasted animals [6,7]. In addition, no intestinal glucose synthesis was observed in post-absorptive or 24 h-fasted rats *in vivo*, especially from [¹⁴C]glutamine [4,5]. By contrast, they concluded from their data that the small intestine of the rat accounted for 21, 35, 19 and 19% of systemic gluconeogenesis after 48 or 72 h of fasting, after 3 days of Type 1 diabetes and 2–15 days of a high-protein diet respectively [4–7]. These authors also found that, in the rat small intestine, an increase in the expression and activity of PEPCK (phosphoenolpyruvate carboxykinase) and glucose-6-phosphatase, two key gluconeogenic enzymes, was consistent with the *in vivo* induction of gluconeogenesis [5,10,11]. Furthermore, they claimed that the

satiety induced by a high-protein diet was linked to the increase in intestinal gluconeogenesis caused by such a diet [6]. Thus, in the light of the latter studies, intestinal gluconeogenesis would become a central process controlling not only glucose homeostasis but also the whole energy metabolism of the body.

The observations and conclusions of Mithieux and co-workers [4,7] are intriguing because, unlike the liver and the kidney, the small intestine of the rat has until recently never been considered to be a site of intense gluconeogenic capacity. To our knowledge, only Windmueller and Spaeth [12,13] previously observed *in vivo* that the small intestine of the adult rat has a very low capacity to synthesize radioactive glucose from radioactive aspartate, glutamine and lactate. *In vitro* preparations of the small intestine from suckling but not weaned rats have also been reported to synthesize some glucose from lactate [14].

Given (i) the potential importance of the small intestine as a gluconeogenic organ and as the source of major glucose homeostasis disturbances, (ii) the absence of glucose synthesis from glutamine in isolated enterocytes from fed rats shown by Watford et al. [8] and Baverel and Lund [9], (iii) that the conclusions drawn by Mithieux and co-workers [4,7] should be taken with caution, as pointed out by the authors themselves because of inaccuracies inherent to the numerous experimental steps needed to determine the gluconeogenic rates they reported [15], and (iv) the recent review by Watford [16] casting serious doubt on the existence of intestinal gluconeogenesis, we have decided to thoroughly re-examine this question both *in vitro* and *in vivo*. For this, we have isolated metabolically viable segments of the small intestine obtained from rats subjected to a 72 h fast, an experimental condition reported so far to be the most potent inducer of intestinal gluconeogenesis [5]. These segments, made of the entire intestinal wall to mimic *in vitro* the structure of the small intestine *in vivo*, were incubated with ¹³C-labelled glutamine. Taking advantage of the ¹³C NMR spectroscopy and enzymatic approaches

Abbreviation used: PEPCK, phosphoenolpyruvate carboxykinase.

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that enabled us to unravel the complexity of the renal metabolism of various substrates [17–22], we measured glutamine utilization and product accumulation. Despite high rates of glutamine metabolism, substantial glucose synthesis could not be detected. Furthermore, arteriovenous concentration difference measurements were performed across the intestine not only in 72 h-fasted Wistar but also in 72 h-fasted Sprague–Dawley rats because the latter rat strain was used by Mithieux and co-workers [4–7]. The results obtained revealed that the small percentage of glutamine taken up by the intact intestine cannot explain the large glucose production reported by Mithieux and co-workers in their rats under the same experimental conditions. A preliminary account of part of the present results has been published in abstract form [23].

METHODS

Reagents

Glucosamine and glutaminase (grade V) were from Sigma (St Quentin-Fallavier, France). Other enzymes and coenzymes were purchased from Boehringer Mannheim (Meylan, France). L-[3-¹³C]Glutamine and D-[2-¹³C]glucose were obtained from Eurisotop (Saclay, France) and had a 99 % isotopic abundance.

Rats

All experiments were approved by the Institutional Animal Care and Use Committee of the Lyon 1 University. Male Wistar or Sprague–Dawley rats weighing between 250 and 300 g were obtained from Charles River (Saint-Germain-sur-l'Arbresle, France). They were acclimated to our animal house for 1 week, kept on a 12 h light/12 h dark cycle and fed a standard diet (U.A.R., Villemoisson-sur-Orge, France). Then, they were fasted for 72 h before the experiments but had free access to water.

Preparation and incubation of intestinal segments from 72 h-fasted Wistar rats

Each rat was anaesthetized with intraperitoneal Nembutal (50 mg · g body weight⁻¹). The abdomen was opened and the small intestine was exteriorized, quickly excised and stripped away from the mesentery. The lumen of the small intestine was flushed out with twice 60 ml of ice-cold oxygenated Krebs–Henseleit medium [23a]. All the subsequent steps of preparation were performed in cold oxygenated Krebs–Henseleit buffer. Then, rings of 2–4 mm width were cut with scissors from the entire length of the small intestine. The rings were pooled and randomized in a flask and then separated from the buffer on a sieve, gathered on a cold glass surface and divided into five equivalent batches of rings that were immediately incubated. Two batches of rings were incubated per incubation time (20 and 30 min) in 250 ml Erlenmeyer flasks containing an atmosphere of O₂/CO₂ (19:1) and 20 ml of Krebs–Henseleit buffer without or with 5 mM L-[3-¹³C]glutamine, 1 or 5 mM unlabelled or D-[2-¹³C]glucose. Glucosamine (40 mM), an inhibitor of hexokinase [24], was also used in certain experiments. The flasks were incubated at 37 °C in a shaking water bath. Incubation was terminated by the addition of HClO₄ [final concentration 5 % (v/v)] and rapid homogenization of tissue plus medium. A batch of rings was taken to determine the initial amounts of added and endogenous substrates by adding HClO₄ before the rings. After centrifugation (3000 g for 5 min), the denatured rings were used for measurement of their protein content; the supernatant was neutralized with a mixture of 20 % (w/v) KOH and 1 % (v/v) H₃PO₄ and used for metabolite determination and NMR spectroscopy.

Measurement of arteriovenous concentration differences

Anaesthetized, 72 h-fasted Wistar and Sprague–Dawley rats (250–300 g) were placed on a heated table to maintain their body temperature at 37–38 °C. Then, a catheter was inserted into the carotid artery. After laparotomy, the gut was gently moved on the left side and covered with gauze moistened with warm saline. Hepatic portal blood was collected in the following way: a needle (gauge 23) was bent, mounted on a pipette holder, connected to a syringe by a length of polyethylene tubing and introduced into the portal vein by puncture. For blood withdrawing, the position of the needle tip inside the hepatic portal vein was continuously controlled thanks to a binocular lens with a magnification of × 8. Blood samples (1.5 ml each) were aspirated slowly (over 60 s) and simultaneously from the carotid artery and the hepatic portal vein. This procedure was never accompanied by any bleeding. Immediately after blood collection, 1.3 ml of blood sample was deproteinized with 0.5 ml of 20 % (v/v) chilled HClO₄. After centrifugation, the supernatants were neutralized with a mixture of KOH and phosphoric acid as described above and used for metabolite analysis.

Analytical methods

ATP and protein content

The intestinal tissue (rings) ATP concentration was quantified by using the method of Lamprecht and Trautschold [25]. Pellets were solubilized in 0.5 M NaOH for protein determination and total protein was determined as described previously [26].

Metabolite assays

Glutamine, glutamate, alanine, lactate, pyruvate, aspartate, glucose, ammonia, glycerol, D-β-hydroxybutyrate, acetoacetate and glycogen were determined according to the method of Passonneau and Lowry [27].

¹³C NMR techniques

Data were recorded as indicated previously [17,18,22] at 125.75 MHz on a Bruker AM-500 WB spectrometer using a 5 mm broadband probe thermostatically maintained at 8 ± 0.5 °C, except that the number of scans was 420. Chemical shifts were expressed as p.p.m. (parts per million) relative to tetramethylsilane. Assignments were made by comparing the chemical shifts obtained with those given in the literature [28,29]. Given that glutamine gluconeogenesis from [3-¹³C]glutamine leads to the equal labelling of the C-1, C-2, C-5 and C-6 of glucose [21], and taking a signal/noise ratio equal to 1.5 for the heights of the corresponding β resonances of glucose carbons, our calculations indicate that, under our experimental conditions, approx. 0.7 μmol of the C-3 of [3-¹³C]glutamine had to be converted into glucose to enable us to detect glutamine gluconeogenesis.

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 (20 or 30 min) per g of intestinal tissue protein.

With [3-¹³C]glutamine as substrate, the transfer of the C-3 of glutamine to a given position in a given metabolite was calculated by using the formula described previously [17,18,22].

The blood metabolite concentrations found in the carotid artery and the hepatic portal vein were compared using the

Table 1 Metabolism of 5 mM L-[3-¹³C]glutamine in small intestinal segments from 72 h-fasted rats

Intestinal segments (267 ± 14 mg of protein per flask) were incubated for 20 and 30 min as described in the Methods section. Results for metabolite removal (–) or production (measured enzymatically and given in $\mu\text{mol} \cdot \text{g}$ of protein^{–1} · incubation time^{–1}) are reported as means $\pm$ S.E.M. for four experiments. The ¹³C NMR data corresponding to these experiments are reported in Table 2.

Incubation time	Added substrate	Metabolite removal (–) or production								
		Glutamine	Glutamate	Alanine	Lactate	Pyruvate	Aspartate	Glucose	NH ₄ ⁺	Urea
20 min	5 mM Glutamine	-105.8 ± 7.7	27.3 ± 2.9	49.9 ± 7.4	18.2 ± 2.4	2.0 ± 0.3	2.5 ± 1.5	0.3 ± 0.1	75.3 ± 13.7	4.0 ± 1.9
	None	-1.5 ± 1.3	4.8 ± 0.2	16.3 ± 2.6	6.9 ± 1.9	1.2 ± 0.2	0.2 ± 0.7	-0.03 ± 0.04	16.4 ± 4.8	3.0 ± 1.6
30 min	5 mM Glutamine	-156.6 ± 12.1	37.4 ± 4.8	114.3 ± 33.1	30.9 ± 3.9	3.4 ± 0.4	3.5 ± 2.0	0.3 ± 0.1	133.8 ± 25.8	6.3 ± 1.6
	None	-0.8 ± 2.0	8.5 ± 1.4	21.7 ± 3.1	15.2 ± 4.2	1.3 ± 0.2	0.8 ± 0.6	0.1 ± 0.1	16.9 ± 8.0	6.6 ± 2.4

paired Student's *t* test. *P* < 0.05 was considered to be statistically significant.

RESULTS

Glutamine metabolism in intestinal segments from 72 h-fasted rats

Since the viability of isolated rat enterocytes is limited with time [8], our segments were incubated for 20 and 30 min, but, for accuracy purposes, these short incubation times were compensated by the incubation of large amounts of intestinal tissue in a large volume (20 ml) of incubation medium. At zero time, the ATP content of intestinal segments was $2.99 \pm 0.71 \mu\text{mol} \cdot \text{g}$ of protein^{–1} (*n* = 3). In intestinal segments incubated with glutamine, the ATP content was 2.90 ± 0.49 and $2.90 \pm 0.39 \mu\text{mol} \cdot \text{g}$ of protein^{–1} after 20 and 30 min of incubation respectively (*n* = 3).

Table 1 shows the time course of glutamine utilization and product formation measured by enzymatic methods when the segments were incubated with 5 mM [3-¹³C]glutamine as substrate. Glutamine utilization occurred at high rates and in a linear fashion with time. The rate of glutamate accumulation was approximately constant, whereas those of alanine, lactate and ammonia tended to increase with time. Small amounts of aspartate, urea and pyruvate accumulated and negligible amounts of glucose were synthesized. In the absence of glutamine, substantial amounts of alanine, lactate and ammonia, and very small amounts of glucose, accumulated from endogenous substrates (Table 1).

Figure 1(A) shows a representative ¹³C NMR spectrum of perchloric extracts obtained after 30 min of incubation of intestinal segments from a 72 h-fasted rat with 5 mM [3-¹³C]glutamine. This spectrum, in which all the most significant resonances could be identified, shows the main non-volatile carbon products of glutamine metabolism. Not only glutamate but also alanine and lactate were the most important labelled products found. The fact that the C-2 of glutamate became labelled during incubation indicates that glutamate was re-synthesized after passage of the C-3 of glutamine through the tricarboxylic acid cycle. Small amounts of the C-3 of glutamine were also incorporated into aspartate, proline and ornithine. No labelling of glucose carbons was observed, as shown in the upper left panel of Figure 1(A) showing the baseline of the spectrum where the glucose resonances appear (in the 100–60 p.p.m. region).

From the spectra obtained after 20 and 30 min of incubation of segments from 72 h-fasted rats, we calculated the amounts of [3-¹³C]glutamine removed and of labelled products accumulated after correction for the ¹³C natural abundance. As shown in Table 2, the removal of the labelled glutamine was also approximately linear with time and this removal was not statistically different with that measured enzymatically (see Table 1). This means that, in agreement with the very low activity of glutamine

synthetase in the small intestine [30], there was no substantial glutamine synthesis in our experiments. The labelling of glutamate, alanine and lactate was lower than the amounts of these products found to accumulate (see Table 1); this was especially true for alanine and to a lesser extent for glutamate and lactate. The fact that the labelling of aspartate was higher than the net aspartate accumulation presented in Table 1 simply reflects the fact that the unlabelled aspartate brought by the segments at zero time was metabolized and replaced by newly synthesized labelled aspartate. Table 2 also shows that the labelled C-2 of aspartate, alanine and lactate was virtually equal to the labelled C-3 of these compounds; this is in agreement with the view that the C-3 of glutamine converted into the C-3 of glutamate by glutaminase and then into the C-3 of 2-oxoglutarate by alanine, aspartate and ornithine aminotransferases, was further metabolized in the tricarboxylic acid cycle via succinate and fumarate, two symmetrical molecules. The passage through these symmetrical molecules is also consistent with the labelling of the C-2 of glutamate which involves one complete turn of the tricarboxylic acid cycle. The labelling of the C-1 of lactate and alanine is also consistent with the recycling of the C-3 of glutamine through the tricarboxylic acid cycle to yield the C-1 of malate and then the C-1 of pyruvate by malic enzyme, or the C-1 of oxaloacetate and then the C-1 of pyruvate by oxaloacetate decarboxylase or PEPCK plus pyruvate kinase [8]. In agreement with the results of Windmueller and Spaeth who used [¹⁴C]glutamine as substrate [13,31,32], some ¹³C-labelled ornithine, citrulline and proline were also formed from ¹³C-labelled glutamine. It is noteworthy that the sum of the non-volatile labelled products of [¹³C]glutamine metabolism increased linearly with time. Neglecting the possibility that a small fraction of the [¹³C]glutamine removed was incorporated into tissue materials [13,31,32], we calculated the ¹³CO₂ produced from [3-¹³C]glutamine to estimate the complete oxidation of glutamine carbon skeletons, as explained in a previous publication [21]. After both 20 and 30 min of incubation, approximately half of the C-3 of glutamine used was converted into CO₂ (59.4 ± 8.9 and $81.2 \pm 11.2 \mu\text{mol} \cdot \text{g}$ of protein^{–1} · incubation time^{–1} respectively). The corresponding proportions of glutamine carbon converted into CO₂ are close to those found in the rat small intestine *in vivo* [13,31,32], indicating that all steps of the tricarboxylic acid cycle were functioning well in our segments *in vitro*.

Evidence that gluconeogenesis from glutamine in small intestinal segments from 72 h-fasted Wistar rats was not masked by concomitant glucose re-utilization

Given the high capacity of the small intestine to metabolize glucose *in vitro* [8,33,34], we used two experimental approaches to test the possibility that the [¹³C]glucose synthesized from part of the [3-¹³C]glutamine metabolized by our intestinal fragments was re-utilized via the glycolytic pathway. As done in a recent study

Figure 1 ^{13}C NMR spectra (125.17 MHz) of neutralized HClO_4 extracts obtained from small intestinal segments (from 72 h-fasted Wistar rats) incubated for 30 min with $[3\text{-}^{13}\text{C}]\text{glutamine}$ in the absence (A) and the presence (B) of glucosamine

(1) Alanine C-3 (17.42); (2) lactate C-3 (21.45); (3) unidentified and unmetabolized glutamine C-3 contaminant (26.41); (4) glutamine C-3 (27.06 and 27.20 and 27.33); (5) glutamate C-3 (28.01 and 27.88 and 27.74); (6) ornithine C-3 (28.41); (7) citrulline C-3 (28.77); (8) proline C-3 (30.07); (9) glutamine C-4 (31.88 and 32.16); (10) glutamate C-4 (34.88); (11) aspartate C-3 (37.57); (12) unidentified (38.57); (13) glycine C-2 (42.48 and 42.70 and 42.91) added as internal standard; (14) unidentified (48.52); (15) artefact generated by the spectrometer in the presence of Krebs–Henseleit buffer and/or biological samples (50.18); (16) alanine C-2 (51.64); (17) aspartate C-2 (53.09); (18) glutamine C-2 (55.10 and 55.38); (19) glutamate C-2 (55.62); (20) lactate C-2 (69.51); (21) glycine C-1 (173.49 and 173.70 and 173.91); (22) glutamine C-1 (175.23); (23) glutamate C-1 (175.71); (24) alanine C-1 (177.03); (25) glutamine C-5 (178.62); (26) glutamate C-5 (182.48); (27) lactate C-1 (183.70). Glucosamine resonances are indicated by the upper-case letter 'G'. The chemical shifts are: 55.28; 57.80; 61.43; 61.56; 63.45; 63.96; 70.54; 70.67; 72.03; 72.06; 72.61; 72.88; 74.39; 75.31; 77.05; 90.11; 93.63; 143.07; 143.20; 145.36; 154.22; 154.75; 155.88.

Table 2 Metabolism of 5 mM L-[3-¹³C]glutamine in small intestinal segments from 72 h-fasted rats

Intestinal segments (267 ± 14 mg of protein per flask) were incubated for 20 and 30 min as described in the Methods section. Results for ¹³C-labelled metabolite removed (–) or accumulated (given in $\mu\text{mol} \cdot \text{g of protein}^{-1} \cdot \text{incubation time}^{-1}$) are reported as means $\pm$ S.E.M. for four experiments. [3-¹³C]Glutamine removal was calculated as the difference between the values found in zero-time flasks and those in incubated flasks. Substrate utilization and product formation measured enzymatically are reported in Table 1.

Incubation time	Amount of labelled metabolites ($\mu\text{mol} \cdot \text{g of protein}^{-1}$)													
	Glutamine		Glutamate		Ornithine	Proline	Citrulline		Aspartate		Alanine		Lactate	
	C-3	C-3	C-2	C-3	C-3	C-3	C-2	C-3	C-1	C-2	C-3	C-1	C-2	C-3
20 min	–104.7 $\pm$ 15.5	18.8 $\pm$ 2.5	1.3 $\pm$ 1.0	0.6 $\pm$ 0.1	0.5 $\pm$ 0.2	0.6 $\pm$ 0.4	1.5 $\pm$ 0.2	1.5 $\pm$ 0.1	0.5 $\pm$ 0.2	6.3 $\pm$ 0.6	6.0 $\pm$ 0.7	0.4 $\pm$ 0.1	3.7 $\pm$ 0.6	3.6 $\pm$ 0.5
30 min	–162.4 $\pm$ 17.9	25.8 $\pm$ 4.6	1.8 $\pm$ 1.1	0.2 $\pm$ 0.1	1.0 $\pm$ 0.3	1.8 $\pm$ 0.6	2.7 $\pm$ 0.1	2.4 $\pm$ 0.1	1.1 $\pm$ 0.4	14.2 $\pm$ 1.2	13.7 $\pm$ 1.3	0.8 $\pm$ 0.1	8.1 $\pm$ 0.6	7.6 $\pm$ 0.5

performed with precision-cut human kidney slices [22], we hypothesized that the [3-¹³C]glutamine not accounted for by the non-volatile ¹³C-labelled products found to accumulate might have been initially converted into [¹³C]glucose and then re-utilized and oxidized into ¹³CO₂ by our intestinal segments. It can be calculated that, if all this synthesized [¹³C]glucose had accumulated in the incubation medium, its concentration would have been approx. 1 mM.

Effect of glucosamine on the metabolism of 5 mM [3-¹³C]glutamine

First, we tested the efficacy of glucosamine, an inhibitor of hexokinase [24], on glucose utilization by small intestinal segments from 72 h-fasted Wistar rats. With 1 mM D-[2-¹³C]glucose as substrate, addition of 40 mM glucosamine induced a mean 34 % inhibition of the removal of [2-¹³C]glucose, measured by NMR spectroscopy; the corresponding values were 55.7 ± 1.5 and $36.6 \pm 1.8 \mu\text{mol} \cdot \text{g of protein}^{-1} \cdot 30 \text{ min}^{-1}$ in the absence and the presence of glucosamine respectively ($n = 3$; $P < 0.05$).

Figure 1(B) shows a ¹³C NMR spectrum obtained in the presence of 40 mM glucosamine in one representative experiment out of two performed in duplicate in which small intestinal segments from 72 h-fasted Wistar rats were incubated for 30 min with 5 mM [3-¹³C]glutamine in the absence and the presence of 40 mM glucosamine. This spectrum, which shows the resonances corresponding to the C-3 of glutamine and those of the ¹³C-labelled products of [¹³C]glutamine (as in Figure 1A) and the resonances of the glucosamine carbons, clearly demonstrates that there were no glucose carbon resonances. In this respect, it should be pointed out that the resonances of the C-1 of glucose, which are well separated from those of the C-1 of glucosamine, were absent.

Effect of 5 mM glucose on the metabolism of 5 mM [3-¹³C]glutamine in small intestinal segments from 72 h-fasted Wistar rats

With the hope that large amounts of glucose added at zero time would (i) dilute the ¹³C-labelled glucose possibly synthesized from [3-¹³C]glutamine and (ii) prevent its complete re-utilization, four additional experiments were performed with small intestinal segments (265.8 ± 8.0 mg of protein $\cdot$ flask^{–1}) from 72 h-fasted Wistar rats incubated for 30 min with 5 mM [3-¹³C]glutamine in the absence and the presence of 5 mM unlabelled D-glucose. Under the latter conditions, glutamine utilization was 136.5 ± 8.9 and $142.4 \pm 10.1 \mu\text{mol} \cdot \text{g of protein}^{-1} \cdot 30 \text{ min}^{-1}$ respectively. In the presence of 5 mM glucose, glucose utilization was $148.8 \pm 15 \mu\text{mol} \cdot \text{g of protein}^{-1} \cdot 30 \text{ min}^{-1}$. Figure 2 presents a ¹³C NMR spectrum obtained in the presence of 5 mM [3-¹³C]glutamine + 5 mM unlabelled D-glucose in one representative experiment. This spectrum, which shows the resonance of the C-3

of glutamine and those of the ¹³C-labelled products of [¹³C]glutamine metabolism (as in Figure 1), clearly demonstrates the presence of glucose carbon resonances. However, as suggested by the fact that all the carbon resonances (the sums of the anomers α and β) had virtually the same intensities (peak heights) and peak areas, these resonances corresponded exclusively to the natural abundance of the glucose carbons added at zero time and not metabolized by the intestinal segments after 30 min of incubation. Therefore no synthesis of ¹³C-labelled glucose carbons occurred from [3-¹³C]glutamine in these experiments.

Metabolite concentrations in arterial and portal vein blood of 72 h-fasted Wistar and Sprague–Dawley rats

Table 3 shows the mean concentrations of all the metabolites measured in the arterial and portal vein blood of 72 h-fasted rats. In both strains, there was a net uptake of glucose; this markedly contrasts with the absence of net glucose uptake or release observed by Mithieux et al. [5] in their 72 h-fasted Sprague–Dawley rats. In agreement with the classical results obtained by numerous authors in fed rats or in rats fasted for up to 48 h [13,31,35–38], we observed a 30 % uptake of arterial glutamine by the intestine in our 72 h-fasted Wistar rats, whereas the corresponding value was only 18 % in our 72 h-fasted Sprague–Dawley rats.

A statistically significant release of glutamate occurred only in Sprague–Dawley rats and this release represented 60 % of the glutamine taken up in these animals. A release of alanine, which was of the same order of magnitude in Wistar and Sprague–Dawley rats, was observed.

In agreement with the observation of Windmueller and Spaeth [13] in their overnight-fasted Osborne–Mendel rats when arterial lactate was higher than 1.2–1.6 mM, the intestine of our Wistar rats took up a large fraction (37 %) of circulating lactate; by contrast, the intestine of our Sprague–Dawley rats neither took up nor released lactate in a statistically significant manner. A very small uptake of glycerol occurred only in Sprague–Dawley rats. D- β -Hydroxybutyrate, whose arterial concentration was much higher in Wistar than in Sprague–Dawley rats, was taken up only by the intestine of Wistar rats and part of this D- β -hydroxybutyrate was released as acetoacetate which was neither taken up nor released by the intestine of Sprague–Dawley rats.

DISCUSSION

Although we used Wistar rats after 72 h of fasting, an experimental condition reported *in vivo* to induce a large intestinal gluconeogenesis [5], the results of the present study unequivocally demonstrate that, when incubated *in vitro*, the small intestine of our

Figure 2 ¹³C NMR spectrum (125.17 MHz) of neutralized HClO₄ extracts obtained from small intestinal segments (from 72 h-fasted Wistar rats) incubated with [3-¹³C]glutamine in the presence of unlabelled glucose

Glucose resonances: I α , glucose C-6 α (61.8); I β , glucose C-6 β (61.8); II, glucose C-4($\alpha + \beta$) (70.7); III, glucose C-2 α (72.5); IV, glucose C-5 α (72.6); V, glucose C-3 α (73.9); VI, glucose C-2 β (75.3); VII, glucose C-3 β (76.8); VIII, glucose C-5 β (76.9); IX, glucose C-1 α (93.2); X, glucose C-1 β (97.0). Other resonances are indicated as in Figures 1(A) and 1(B).

Table 3 Arterial and portal vein blood concentrations of metabolites in 72 h-fasted Wistar and Sprague–Dawley rats

n = number of rats. The metabolites were measured as described in the Methods section. Values are means $\pm$ S.E.M. Statistical difference was measured by the paired Student's *t* test for paired data between the arterial and portal vein blood: **P* < 0.05.

Rat strain	Blood vessel	Blood metabolite concentration							
		Glucose (mM)	Glutamine (mM)	Glutamate (mM)	Alanine (mM)	Lactate (mM)	Glycerol (μ M)	D- β -Hydroxybutyrate (mM)	Acetoacetate (mM)
Wistar (<i>n</i> = 6)	Artery	7.51 $\pm$ 0.11	0.567 $\pm$ 0.058	0.173 $\pm$ 0.015	0.328 $\pm$ 0.010	1.67 $\pm$ 0.21	84 $\pm$ 10	1.630 $\pm$ 0.23	0.263 $\pm$ 0.025
	Portal vein	6.25 $\pm$ 0.14*	0.393 $\pm$ 0.029*	0.184 $\pm$ 0.021	0.366 $\pm$ 0.012*	1.05 $\pm$ 0.04*	75 $\pm$ 2	1.160 $\pm$ 0.15*	0.543 $\pm$ 0.058*
	Arteriovenous difference	1.26 $\pm$ 0.17	0.174 $\pm$ 0.074	−0.011 $\pm$ 0.013	−0.038 $\pm$ 0.011	0.62 $\pm$ 0.19	9 $\pm$ 11	0.471 $\pm$ 0.189	−0.281 $\pm$ 0.046
Sprague–Dawley (<i>n</i> = 7)	Artery	8.00 $\pm$ 0.36	0.478 $\pm$ 0.038	0.258 $\pm$ 0.018	0.284 $\pm$ 0.030	1.16 $\pm$ 0.22	85 $\pm$ 11	0.555 $\pm$ 0.155	0.289 $\pm$ 0.085
	Portal vein	6.81 $\pm$ 0.34*	0.393 $\pm$ 0.041*	0.310 $\pm$ 0.015*	0.330 $\pm$ 0.030*	0.91 $\pm$ 0.12	71 $\pm$ 13*	0.541 $\pm$ 0.149	0.310 $\pm$ 0.064
	Arteriovenous difference	1.19 $\pm$ 0.38	0.085 $\pm$ 0.044	−0.052 $\pm$ 0.017	−0.046 $\pm$ 0.010	0.25 $\pm$ 0.15	14 $\pm$ 6	0.014 $\pm$ 0.022	−0.021 $\pm$ 0.045

animals did not synthesize glucose from glutamine, the main if not the exclusive gluconeogenic precursor after 48 h [4] and presumably 72 h of fasting. In that respect, it resembles the isolated perfused and intact functioning small intestine of overnight-fasted rats [31,32] and the enterocytes isolated from fed rats in which no glutamine gluconeogenesis was reported [8,9]. It should be mentioned here that, within the intestinal wall, the enterocytes are the only site of glutamine metabolism [31]. Moreover, our *in vivo* results do not corroborate those of Mithieux et al. [5] who concluded that the small intestine of 72 h-fasted Sprague–Dawley rats is responsible for at least one-third (35 %) of systemic gluconeogenesis.

Metabolic viability of our small intestinal fragments and absence of glucose synthesis

Several lines of evidence indicate that our intestinal segments, which consisted of the entire small intestinal wall, were metabolically viable. Taking a dry weight/fresh weight ratio equal to 0.23 [31] and assuming that 90 % of the dry weight consisted of protein, it can be calculated that the small intestine preparation used by Windmueller and Spaeth [32] metabolized the lumenally added glutamine (6 mM) at a rate of 27 μ mol · g of protein^{−1} · 30 min^{−1}. Taking the glutamine removed in our experiments after 30 min of incubation (Table 1), it can also be calculated that

our small intestinal segments, a maximum of 40 % of which consisted of enterocytes [39,40], metabolized 5 mM glutamine at a much higher rate ($586 \mu\text{mol} \cdot \text{g}$ of protein⁻¹ · 30 min⁻¹). The enterocytes isolated by Watford et al. [8] and by Baverel and Lund [9] metabolized 5 mM glutamine at a rate of 367 and $300 \mu\text{mol} \cdot \text{g}$ of protein⁻¹ · 30 min⁻¹ respectively. Thus, although our measurements and calculations reveal that the ATP level of our segments was only about one-half that found *in vivo* [41], and although the enterocytes represented only approx. 40 % of our intestinal segments, they were metabolically very active and metabolized glutamine avidly and in a virtually linear manner with time (Tables 1 and 2). It is also important to note that, like the glutamine taken up from the lumen and from the circulating blood in the *in vivo* preparations used by Windmueller and Spaeth [13,31], about half of our glutamine metabolized was completely oxidized (see the Results section). This means that the mitochondria of our intestinal segments functioned satisfactorily. That the entire tricarboxylic acid cycle was operating in a satisfactory manner is also indicated by the conversion of the C-3 of glutamine not only into the C-2 and C-3 of alanine and lactate but also into the C-2 of glutamate (Figure 1A and Table 2). Thus the absence of substantial glucose synthesis in these segments was not due to a limitation in the provision of ATP but rather to the intrinsic organization of the glutamine metabolic pathways of rat enterocytes. It should also be mentioned here that Windmueller and Spaeth [32] have concluded from their experiments that the glutamines taken up from the lumen on the one hand and, on the other hand, from blood shared a common metabolic pool. Thus our results obtained *in vitro* with luminally added glutamine can be considered relevant to the *in vivo* situation.

The very small amounts of glucose synthesized, which were not higher in the presence than in the absence of glutamine, presumably originated from the degradation of the very small amount of glycogen present in the intestinal segments of 72 h-fasted rats ($13 \mu\text{mol}$ glucosyl equivalents · entire small intestine⁻¹).

Similarly, the absence of glutamine gluconeogenesis in our intestinal segments was not due to the re-utilization of [¹³C]glucose initially synthesized from [3-¹³C]glutamine as strongly suggested not only by the use of glucosamine, an inhibitor of hexokinase [24], but also by the incubation of [¹³C]glutamine with a physiological concentration (5 mM) of glucose (see Figures 1B and 2). Under the latter experimental condition (see the Results section), a strong line of evidence that no synthesis of [¹³C]glucose from [3-¹³C]glutamine occurred is that the resonance intensities and areas ($\alpha + \beta$ anomers) of the six glucose carbons were equal (see Figure 2 and the Results section); indeed, as recently shown in rat renal proximal tubules, gluconeogenesis from [3-¹³C]glutamine leads to glucose carbons labelled with carbon 13 to different degrees [21]. It should also be mentioned here that enterocytes prepared from the small intestine adapted to a high-protein diet did not produce glucose from glutamine [42].

Arteriovenous concentration difference measurements

To our knowledge, our study is the first to report arteriovenous concentration difference measurements across portal-drained viscera of 72 h-fasted rats. It should be pointed out that, unlike Mithieux et al. [5] in their *in vivo* experiments, we did not ligature the inferior mesenteric artery in order to avoid surgical stress. But this is unimportant with respect to glutamine metabolism because it occurs primarily in the small intestine; indeed, 87 % of glutaminase is located in the small intestine [43] and therefore, as noted by Windmueller and Spaeth [32], most of the glutamine uptake in our rats occurred in the small intestine.

After 72 h of fasting, our observation of a net intestinal uptake of glucose in both Wistar and Sprague–Dawley rats (Table 3) markedly contrasts with the findings of Mithieux and co-workers [4,5] who reported no net production or uptake of glucose by the small intestine of both 48 h- and 72 h-fasted Sprague–Dawley rats. However, this is in agreement with the observation of Burrin et al. [44] who also found no evidence of gluconeogenesis in the small intestine of 36 h-fasted pigs. Since a very small, virtually negligible, glycerol uptake was observed only in Sprague–Dawley rats (Table 3), this means that the small intestine *in vivo* cannot synthesize substantial amounts of glucose from this substrate.

Wistar rats

Considering that two glutamine molecules are needed to synthesize one glucose molecule, and neglecting the fact that about half the circulating glutamine taken up by the intestine was oxidized to CO₂, one may calculate from our data that the arterial glucose entering the intestine would have been diluted by only 1.2 % (0.174:2:7.51) by the glutamine-derived glucose; the corresponding value would have been only 0.6 % if one considers that half the glutamine taken up was oxidized to CO₂. Unfortunately, Mithieux et al. [5] did not report the intestinal substrate uptake in their experiments. In agreement with observations of Windmueller and Spaeth [13] when the blood lactate was elevated, substantial amounts of lactate were taken up by the intestine of our 72 h-fasted Wistar rats, but this potential gluconeogenic substrate could not have been converted into glucose because of the extremely low pyruvate carboxylase activity measured in the small intestine of the adult rat [45,46]. In addition, Mithieux and co-workers [4] did not observe any conversion of [¹⁴C]lactate into [¹⁴C]glucose in the small intestine of 48 h-fasted rats *in vivo*. Furthermore, our preliminary results *in vitro* indicate that 5 mM [¹³C]lactate is not converted into [¹³C]glucose in intestinal segments prepared from 72 h-fasted Wistar rats (results not shown).

Sprague–Dawley rats

It should be pointed out that, in our 72 h-fasted Sprague–Dawley rats, glutamine was the sole potential gluconeogenic precursor taken up in substantial amounts by the intestine (Table 3). Doing the same calculations as above and neglecting the fact that some if not all of the glutamate released originated from glutamine (Table 3), it can be calculated that, if all the glutamine taken up were converted into glucose, this would have diluted the arterial glucose entering the intestinal circulation by 0.53 % (0.085:2:8.00); taking into account that about half of this glutamine was converted into CO₂, the corresponding value would have been only 0.27 %. These values are different from that (7.9 %) published by Mithieux et al. [5] in 72 h-fasted Sprague–Dawley rats. Therefore our results clearly indicate that both in 72 h-fasted Wistar rats and in 72 h-fasted Sprague–Dawley rats, glutamine gluconeogenesis cannot be detected in a reliable manner in the intestine *in vivo*. Since our data (Table 3) together with those of Windmueller and Spaeth [31] indicate that there were no other potential gluconeogenic precursors taken up by the intestine, this means that intestinal gluconeogenesis cannot be detected *in vivo*. In this context, the reasons why Windmueller and Spaeth [12,13] observed that small percentages of [U-¹⁴C]glutamine (4.7 %), [U-¹⁴C]lactate (0.1–0.2 %) and [U-¹⁴C]aspartate (10 %) were found in [¹⁴C]glucose in the small intestine of their animals deserve careful examination. Since these authors had to chromatographically separate the various products resulting from the intestinal metabolism of their ¹⁴C-labelled substrates, their [¹⁴C]glucose fractions might have been contaminated by some other neutral

^{14}C -labelled compounds. In this respect, we used ^{13}C NMR spectroscopy, which avoids metabolite separations. In agreement with this view, Windmueller and Spaeth [13] found that 6.2 % of ^{14}C -labelled D- β -hydroxybutyrate was also converted into [^{14}C]glucose; since ketone bodies cannot be gluconeogenic [47] their observation must have been due to some methodological artefact if one accepts that the small intestine does not synthesize glucose at all.

Reasons for the discrepancy between our *in vivo* results and those of Mithieux and co-workers

First, it should be pointed out that the arteriovenous difference approach has many limitations. Indeed, numerous experimental steps are necessary to get the final values [blood sampling, centrifugation to get the plasma, possible heterogeneity between the plasma and whole blood often neglected, deproteinization and neutralization of the sample, errors inherent to the enzymatic measurements of metabolites, imperfect chromatographic separation of radioactive metabolites, high blood concentration of glucose, radioactive measurements (radioactive disintegration is a random process), small arteriovenous differences multiplied by high blood flow sometimes measured in separate experiments]. Since each step has its own margin of error, the final values may suffer from substantial errors. Bearing this in mind, we believe that the gluconeogenic rates reported by Mithieux et al. [5] in their 72 h-fasted Sprague–Dawley rats are not compatible with the possible uptake of gluconeogenic substrates. Indeed, they calculated an intestinal gluconeogenesis of $16.9 \mu\text{mol} \cdot \text{kg} \text{ body weight}^{-1} \cdot \text{min}^{-1}$. Taking a mean body weight of 270 g and an intestinal blood flow of $5.2 \text{ ml} \cdot \text{min}^{-1}$ [5], this would mean that $0.885 (4.6:5.2) \mu\text{mol}$ of glucose was added to each millilitre of blood passing through the intestine. Since two molecules of glutamine are needed to synthesize one molecule of glucose, their results would imply that $1.77 \mu\text{mol}$ of glutamine (or of other gluconeogenic precursor) was taken up from each millilitre of arterial blood only for the synthesis of glucose and without any complete oxidation of glutamine into CO_2 . Our data, presented in Table 3, indicate that in fact only $0.085 \mu\text{mol}$ of glutamine was taken up from each millilitre of arterial blood in 72 h-fasted Sprague–Dawley rats (Table 3). Therefore compelling experimental evidence is provided that the gluconeogenic rates reported by Mithieux and co-workers are compatible neither with the uptake and availability of glutamine nor with the availability and uptake of other circulating gluconeogenic precursors.

Moreover, taking into account that for each molecule of glucose synthesized two glutamine carbon skeletons should pass through PEPCK, a mandatory enzyme in glutamine gluconeogenesis, it is important to note that the V_{max} of PEPCK measured by Mithieux et al. [5] was severalfold lower than the rates of gluconeogenesis they calculated. This represents another compelling piece of evidence that the high gluconeogenic rates reported by the latter authors are not compatible with the biochemical characteristics of the intestine of their 72 h-fasted Sprague–Dawley rats.

Glycogen stores and glucose release *in vivo*

Our measurements indicate that the glycogen store in the entire small intestine of 72 h-fasted Wistar rats was $13 \mu\text{mol}$ (in glucosyl equivalents). The complete release of this glycogen as glucose via glycogenolysis would have to occur within 3 min to account for the rate of release of newly synthesized glucose reported by Mithieux et al. [5]. Thus glycogen cannot be the source of the glucose calculated to be released *in vivo* by these authors.

Ketone body metabolism

Another interesting observation made in the present study is that, in the 72 h-fasted rat, there were major strain differences that, to our knowledge, have never been reported so far. Indeed, the intestine of 72 h-fasted Sprague–Dawley rats did not remove circulating ketone bodies at all. By contrast, the intestine of 72 h-fasted Wistar rats removed circulating D- β -hydroxybutyrate but released approx. 60 % of it as acetoacetate. Thus these two strains of rat differ from the Mendel–Osborne rats used by Windmueller and Spaeth [13] who found that, after an overnight fast, the intestine took up both D- β -hydroxybutyrate and acetoacetate. Therefore the traditional view that ketone bodies are important energy substrates of the intestine in the fasting state does not necessarily apply to all strains of rat.

In summary, our *in vitro* data clearly demonstrate that metabolically viable segments from the small intestine of 72 h-fasted rats did not synthesize glucose at all despite a high rate of glutamine utilization and metabolism. Our *in vivo* data not only in Wistar but also in Sprague–Dawley rats after 72 h of fasting indicate that the rate of intestinal gluconeogenesis reported by Mithieux et al. [5] in their 72 h-fasted Sprague–Dawley rats can be accounted for neither by the uptake of the gluconeogenic precursors available in the arterial blood, nor by the V_{max} of PEPCK they measured. Moreover, our results demonstrate that glutamine gluconeogenesis would not be detectable by the currently available methods because the dilution of circulating glucose by newly synthesized glucose would be much smaller than the margins of error of the methods employed. Therefore the view that the intestine of the rat is an important site of gluconeogenesis, at least in 72 h-fasted rats, is no longer tenable.

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