Glutamine synthesis is heterogeneous and differentially regulated along the rabbit renal proximal tubule

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Glutamine synthesis, a major process for ammonia detoxification and the control of acid–base balance, occurs from various precursors in suspensions of rabbit proximal tubules. However, no data are currently available on the distribution of glutamine synthesis along the rabbit proximal tubule, and its modulation by changes of substrate concentration. Therefore we have micro-dissected and incubated the three parts (S1, S2 and S3) of rabbit proximal tubules and measured glutamine synthesis from alanine and aspartate. With a physiological concentration of alanine (0.25 mM) or aspartate (0.05 mM), glutamine synthesis in the S1 segment was about half of that in the S2 and S3 segments, and was greater from alanine than from aspartate along the entire

proximal tubule. Elevation of alanine and aspartate concentrations to 5 mM increased glutamine synthesis in both a substrate- and segment-dependent manner. It is concluded that glutamine synthesis occurs from alanine and aspartate along the entire rabbit proximal tubule; however, contrary to what might have been expected on the basis of measurement of glutamine synthetase activity, the basal rate of glutamine synthesis and its adaptation to increased substrate availability are heterogeneous along this nephron segment.

Key words: kidney, metabolism, ammoniagenesis, alanine, aspartate.

INTRODUCTION

It is known that glutamine synthetase (EC 6.3.1.2), the enzyme which catalyses the conversion of glutamate and ammonia into glutamine, is present in the kidney of rat, sheep, guinea pig, rabbit, mouse and hamster, but absent in the kidney of man, dog, cat and pig [1–6]. The activity of this enzyme in the kidney is thought to be a key factor that limits the release of ammonia, generated by the renal cells in herbivorous species or the rat, into both the urine and renal vein. Thus renal glutamine synthesis contributes not only to a detoxifying process that removes ammonia, a potentially toxic compound to the nervous system [7], but also to the regulation of systemic acid–base balance.

In the rat nephron, glutamine synthetase activity [1], mRNAs and protein [8] are restricted to the proximal straight tubule; in the mouse, the glutamine synthetase mRNAs have been localized in the renal cortex [9] and, more specifically, in the proximal tubule [10]. In contrast, in the rabbit nephron, glutamine synthetase is active in the glomerulus, the entire proximal tubule and the distal straight tubule [1]; in the latter species, the highest activity of this enzyme is found in the proximal tubule, where the activity is in the same range in the proximal convoluted, early and late-proximal straight tubules [1].

In agreement with previous findings, suspensions of rabbit proximal tubules, which contain all parts of the proximal tubules, have been shown to synthesize glutamine from alanine [11], aspartate [11–13], glucose plus ammonia [14] and glutamate [15]. However, despite the fact that glutamine synthetase activity was about the same in the three different parts of the rabbit proximal tubule [1], it remains at present unknown whether or not these three different segments of the proximal tubule synthesize glutamine from a given precursor at the same rates. Indeed, measurements of glutamine synthetase activity provide only an estimate for the potential for glutamine synthesis; in addition,

the conversion of substrates, such as alanine, aspartate, glutamate or glucose plus ammonia, into glutamine involves a number of enzymes whose activities may greatly differ from one segment to another along the proximal tubule [16]. This lack of information is surprising, in view of the many efforts devoted to both the study of the metabolic heterogeneity of the nephron and the great importance of renal glutamine synthesis as both a detoxifying process and an acid–base equilibrium control mechanism.

Therefore, in the present study, we have asked the following questions: (1) does glutamine synthesis occur at the same rate in the three different segments of the rabbit proximal tubule, especially when alanine and aspartate are the substrates added at a low physiological concentration; and (2) is glutamine synthesis increased when the concentrations of alanine and aspartate are elevated, since protein absorption and digestion are known to lead to an increase in the circulating levels of all amino acids that are potential sources of ammonia in the kidney?

In order to examine these questions, we have incubated different parts (S1, S2, and S3 respectively) of rabbit proximal tubules in the presence of alanine and aspartate. Glutamate and glutamine, the products of alanine and aspartate metabolism, were measured in the picomolar range with microanalytical methods involving amplification by enzymic cycling. The results obtained demonstrate that the S1, S2 and S3 segments of the rabbit proximal tubule synthesize glutamine from both alanine and aspartate; at the physiological concentration of alanine (0.25 mM) and aspartate (0.05 mM), the S1 segment synthesizes less glutamine than the S2 and S3 segments. Elevation of alanine concentration to a high, non-physiological one (5 mM) led to an increase in glutamine synthesis only in the S1 and S2 segments, whereas elevation of aspartate concentration to 5 mM resulted in an increase in glutamine synthesis that reached about the same levels in the three segments of the proximal tubule.

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MATERIALS AND METHODS

Preparation of segments of rabbit renal proximal tubules

Male New Zealand White rabbits weighing 1.5–2 kg were obtained from the Elevage Scientifique des Dombes, Châtillon-sur-Chalaronne, France. The animals that were fed on a standard diet (U.A.R., Villemoisson-sur-Orge, France) and had free access to water were anaesthetized with intravenous sodium pentobarbital (50 mg·kg⁻¹). The procedure used for the microdissection of rabbit kidney tubular segments was similar to that described by Imbert et al. [17]. Through a mid-line abdominal incision, the left kidney was surgically exposed and perfused *in situ* via an aortic cannula placed just below the left renal artery. After ligating the aorta just above the aortic bifurcation and between the left and right renal arteries, 25 ml of Krebs–Henseleit medium (118 mM NaCl/4.7 mM KCl/2.5 mM CaCl₂/1.2 mM KH₂PO₄/1.2 mM MgSO₄/25 mM NaHCO₃) warmed to 37 °C and gassed with 95% O₂/5% CO₂ was used to wash the left kidney. Then 15 ml of the same medium containing 1 mg·ml⁻¹ collagenase was perfused over a 2 min period. After ligation of the renal vein and the ureter, 10 ml of this medium was injected until the point at which the renal capsule ruptured; then the kidney was removed and placed in a 154 mM NaCl solution at 0–4 °C. The rabbit was killed by an overdose of intravenous sodium pentobarbital (5 ml of a solution of 0.03 g·ml⁻¹).

Thin pyramid slices were cut with a Stadie–Riggs microtome from the excised kidney and used to prepare tissue pieces from both the cortex and the outer stripe of the outer medulla. These pieces of cortex and medulla were incubated in Krebs–Henseleit medium containing BSA (0.02%, w/v) and collagenase (1 mg·ml⁻¹) in an atmosphere of 95% O₂/5% CO₂ for 10 and 20 min respectively. At the end of the incubation, the tissue pieces were rinsed with collagenase-free dissection solution that consisted of Krebs–Henseleit buffer and 10 mM Hepes, both at pH 7.4. This solution was gassed with 95% O₂/5% CO₂ before addition of 0.02% (w/v) BSA (fraction V, fatty-acid free).

Microdissection of nephron segments was performed at 4 °C with thin needles under stereo-microscopic observation at 25–40× magnification. S1 and S2 segments of the proximal tubule were dissected from the cortical tissue pieces, and the S3 segments were dissected from the outer medulla tissue pieces. The S1 segments, which comprise the beginning and the middle portions of the convoluted part, were selected from proximal tubules microdissected with their glomeruli attached; they were cut close to the glomerulus, and 1–5 mm further down the tubule. The S2 segments comprise the final portions of the convoluted part and the beginning portions of the straight part. In our study, only the straight part was microdissected. After isolation, each microdissected segment was aspirated with 2 µl of dissection medium and transferred to a small disc of dry BSA on a microscope slide, which was cooled to 4–6 °C by a thermostatic system fixed on to the plate of a microscope. The tubule length was measured with an ocular micrometer and hair-tip tools [18] were used to uncoil the tubules. Thereafter, the tubules were placed back into 50 µl of dissection medium in individual wells of a microplate, and kept at 4 °C before incubation. For each animal, the number of nephrons microdissected was between 4 and 32 for S1, S2 or S3.

Incubations

‘Thermowell’ polycarbonate microplates were used for the incubations. Strips of 24 wells were cut off. Each tubule segment was drawn off with 3 µl of dissection medium into the tip of a Drummond variable-stroke piston pipette. The tubule segments

and fluid were deposited from the pipette into the individual cavities of the microplate. Then 5 µl of Krebs–Henseleit medium, pH 7.4, containing 10 mM Hepes, pH 7.4, in the presence or absence of L-alanine or L-aspartate was added to each droplet containing the tubules, after which the microplate was placed inside a small waterproof box, which was immersed in a thermostatically controlled water bath at 37 °C. In order to prevent evaporation, two sheets of filter paper soaked in distilled water were placed in the incubation box. An atmosphere of 95% O₂/5% CO₂ saturated with water at 37 °C was maintained inside the incubation box throughout each experiment. This allowed us to limit the evaporation of fluid to only 0.3–0.4 µl (out of a total volume of 8 µl of incubation medium) after 90 min of incubation at 37 °C. At both zero time and the end of the incubations, 5 µl of 50 mM NaOH was added to each sample used subsequently for metabolite assays.

Analytical methods

The specific methods employed to measure the minute amounts of glutamate and glutamine present either at time zero or the end of the incubation period were adapted from those described by Curthoys and Lowry [19]. These take advantage of the enzymic cycling method developed by Kato et al. [20], which allows an accurate analysis of metabolites in the picomolar range. The microassays were performed in 96-well microplates under mineral oil to minimize evaporation.

Glutamate analysis

The method is on the basis of the conversion of glutamate into 2-oxoglutarate by glutamate dehydrogenase. Aliquots (10 µl) of a solution containing 1 mM NAD⁺, 0.4 mM ADP, 250 mM Tris/acetate buffer, pH 8.4, and 200 µg·ml⁻¹ glutamate dehydrogenase was added to each sample. After 30 min incubation at room temperature, a 9 µl aliquot from each sample was added to 25 µl of 0.1 M NaOH in the wells of a 96-well microplate, and the microplate was heated in an oven at 75 °C for 30 min to destroy the NAD⁺ in excess. Then, an aliquot (10 µl) was extracted to measure the NADH produced by the glutamate dehydrogenase reaction. In order to achieve the required sensitivity, an enzymic cycling procedure was used [20].

Glutamine analysis

The remainder (14 µl) of each original sample in the 96-well microplate was used to measure glutamine. Each sample was first acidified by the addition of 2 µl of 1.5 M HCl to destroy the NADH formed by the glutamate dehydrogenase reaction. Then, 10 µl of a solution containing 0.6 mM EDTA, 500 mM sodium acetate and 75 µg·ml⁻¹ *Escherichia coli* glutaminase was added. After 45 min incubation at 37 °C, 2 µl of 2 M NaOH was added, followed by 10 µl of a solution containing 1 mM NAD, 1 mM ADP, 0.5 M Tris/acetate buffer, pH 8.4, and 300 µg·ml⁻¹ glutamate dehydrogenase. After 30 min of reaction at room temperature, 50 µl of 0.2 M NaOH was added and the samples were heated at 75 °C for 30 min to destroy the NAD in excess. This step was followed by an enzymic cycling procedure used to measure the NADH produced by the glutamate dehydrogenase reaction, monitoring the conversion of glutamine into glutamate.

It should be mentioned that the above general procedure was used when the amounts of glutamate and glutamine expected to be found were 10–40 and 30–125 pmol respectively. An adaptation of this procedure was required when the expected amounts of glutamate and glutamine were lower, i.e. in time zero samples

Figure 1 Time course of glutamine production and glutamate accumulation from 0.25 mM alanine and 0.05 mM aspartate by different proximal tubular segments (S1, S2 and S3)

The Figure shows the time course of glutamine production (■) and glutamate accumulation (□) from 0.25 mM alanine (upper panels) and 0.05 mM aspartate (lower panels) by different proximal tubular segments (S1, S2 and S3 respectively). Values are means $\pm$ S.E.M. for 9–28 segments at each time point in a representative experiment.

or after incubation of the tubules in the absence of alanine and aspartate, or in the presence of a low alanine or aspartate concentration (0.25 or 0.05 mM respectively). This adaptation mainly consisted of a reduction in the volume of the solution used in each analytical step. Standard solutions of glutamate and glutamine were processed in the same way as the tubule samples.

Materials

L-Alanine, L-aspartate, L-glutamine, L-glutamate and glutaminase (grade V) were from Sigma Aldrich Chemie (L'Isle d'Abeau, France). Other enzymes and coenzymes were purchased from Boehringer Mannheim (Meylan, France).

Calculations

Glutamine synthesis and glutamate accumulation, reported as means $\pm$ S.E.M., are expressed in pmol/mm of proximal tubular segment per unit time. Statistical differences in the rates of glutamine synthesis and glutamate accumulation among the three proximal tubular segments studied or, for a given segment, between the two alanine or aspartate concentrations used, were measured by the analysis of variance test followed by the Newman Keul post-hoc test. These tests allowed us to take into account, in the statistical analysis of our results, not only the inter-individual variability of glutamine synthesis and glutamate accumulation (presented as means $\pm$ S.E.M.), but also the intra-individual variability of these parameters measured in 4–32 S1,

S2 or S3 segments in each experiment. The same tests were used to compare (i) both the glutamine synthesized and the glutamate accumulated from a physiological concentration of alanine (0.25 mM) with that from a physiological concentration of aspartate (0.05 mM), and (ii) the glutamine synthesized and the glutamate accumulated from 5 mM alanine with that from 5 mM aspartate.

RESULTS

Figure 1 shows that when alanine or aspartate was the substrate at its respective physiological concentration or at 0.25 mM or 0.05 mM, the three different segments (S1, S2 and S3) of rabbit renal proximal tubules synthesized glutamine in an almost linear manner with time over a 60 min incubation period; this was not always true for glutamate accumulation. When alanine or aspartate concentration was raised to 5 mM, glutamine production was also linear with time over an incubation period of up to 60 min (results not shown).

As clearly shown in Figures 2 and 3, the production of glutamine and the accumulation of glutamate in the presence of either 0.25 mM alanine or 0.05 mM aspartate as the substrate were linear with the length of the S1, S2 and S3 segments. This also held true when alanine or aspartate concentration was 5 mM (results not shown).

Table 1 presents the amounts of glutamine and glutamate found at the end of the incubation period when S1, S2 and S3 segments of rabbit renal proximal tubules were incubated for

Figure 2 Glutamine production and glutamate accumulation from 0.25 mM alanine as a function of the length of different proximal tubular segments (S1, S2 and S3)

Values shown are from a representative experiment. Each point corresponds to one tubular segment; the incubations lasted for 60 min.

60 min in the absence or presence of either 0.25 mM or 5 mM alanine as substrate. In the absence of alanine, the amounts of glutamine and glutamate found were low in the three different segments of the proximal tubules (Table 1). Because, at time zero (the beginning of the incubation period), the amounts of glutamine found were 1.2 ± 0.2 , 1.0 ± 0.1 and 2.7 ± 0.4 pmol·mm⁻¹ in the S1, S2 and S3 segments respectively, this indicates that the rate of glutamine synthesis from endogenous substrates was very low. The amounts of glutamate present at time zero in the S1, S2 and S3 segments were 2.4 ± 0.4 , 4.5 ± 0.8 and 5.2 ± 0.7 pmol·mm⁻¹ respectively. Therefore no net glutamate accumulation occurred from endogenous substrates in these segments after 60 min of incubation (see Table 1).

Discounting the small amounts of glutamine present at time zero, the rate of glutamine synthesis from 0.25 mM alanine in the S1 segments was about half of that observed in the S2 and S3 segments, whereas the accumulation of glutamate found, which increased from the S1 to the S3 segment, was severalfold lower than glutamine synthesis in the three proximal tubular segments.

With a high concentration of alanine (5 mM), the mean rate of glutamine synthesis statistically increased by 69% in the S1 segments ($P < 0.05$), by 36% in the S2 segments ($P < 0.05$), but remained unchanged in the S3 segments when compared with the rates observed with 0.25 mM alanine as substrate. Similarly,

the accumulation of glutamate (which was severalfold lower than glutamine synthesis in the three segments studied) statistically increased only in the S1 and S2 segments ($P < 0.05$) when alanine concentration was raised from 0.25 to 5 mM.

Table 2 presents the amounts of glutamine and glutamate found at the end of the incubation period when S1, S2 and S3 segments of rabbit proximal tubules were incubated for 60 min in the presence of 0.05 or 5 mM aspartate. Discounting the small amount of glutamine present at time zero that has been mentioned above, the rate of glutamine synthesis from 0.05 mM aspartate in the S1 segment was approximately half of that found in the S2 and S3 segments (Table 2); in this case, the glutamate concentrations found, which were lower than the glutamine concentration found in all segments, were similar in the S1, S2 and S3 segments.

When compared with the rates observed in the presence of 0.05 mM aspartate, the means for the rates of glutamine synthesis in the presence of 5 mM aspartate increased by 340% in the S1 segments ($P < 0.05$), 104% in the S2 segments ($P < 0.05$) and 92% in the S3 segments ($P < 0.05$) (Table 2). In this case, the rates of glutamine synthesis were similar in the three different segments. Again, discounting the glutamate found at time zero, it can be seen in Table 2 that elevation of aspartate concentration from 0.05 to 5 mM also increased the glutamate accumulation by

Figure 3 Glutamine production and glutamate accumulation from 0.05 mM aspartate as a function of the length of different proximal tubular segments (S1, S2 and S3)

Values shown are from a representative experiment. Each point corresponds to one tubular segment. The incubation lasted for 60 min.

Table 1 Glutamine and glutamate synthesis in rabbit proximal tubular segments (S1, S2 and S3) after 60 min incubation with alanine

Results (pmol · mm⁻¹ · h⁻¹) are reported as means ± S.E.M. for 5, 7 and 8 experiments (rabbits) in the absence or the presence of 0.25 or 5 mM alanine respectively. *n*, number of rabbits; number of tubular segments per experiment are shown in parentheses. Significance of differences ($P < 0.05$) were calculated by the analysis of variance test, followed by the Newman Keul post-hoc test: *, between S1 and S2; **, between S2 and S3; ***, between S3 and S1 inside the same substrate concentration group.

Segment...	No added substrate (<i>n</i> = 5)			Alanine [0.25 mM] (<i>n</i> = 7)			Alanine [5 mM] (<i>n</i> = 8)		
	S1 (6–15)	S2 (5–15)	S3 (4–12)	S1 (10–23)	S2 (10–28)	S3 (10–24)	S1 (10–24)	S2 (10–24)	S3 (9–24)
Glutamine									
Mean	2.1	2.8	6.2	23.8	48.3	55.1	43.5	65.8	50.3
± S.E.M.	1.1	1.2	1.8	4.3	7.5	7.9	4.5	5.5	5.6
$P < 0.05$				*		***	*	**	
Glutamate									
Mean	2.4	3.3	4.1	5.0	9.4	13.5	10.1	16.2	15.7
± S.E.M.	0.3	0.8	0.8	0.6	1.1	2	2.1	2	2.2
$P < 0.05$				*	**	***			
Glutamine plus glutamate									
Mean	4.5	6.1	10.3	28.8	57.7	68.6	53.7	82.0	66.0
± S.E.M.	1.1	1.1	1.7	4.4	6.8	6.3	5.3	5.7	6.1
$P < 0.05$		**	***	*		***	*		

Table 2 Glutamine and glutamate synthesis in rabbit proximal tubular segments (S1, S2 and S3) after 60 min incubation with aspartate

Results ($\text{pmol} \cdot \text{mm}^{-1} \cdot \text{h}^{-1}$) are reported as means $\pm$ S.E.M. for 6 or 9 experiments (rabbits) in the presence of 0.05 or 5 mM aspartate respectively. n = number of rabbits; number of tubular segments per experiment are shown in parentheses. Significance of differences ($P < 0.05$) were calculated by the analysis of variance test, followed by the Newman Keul post-hoc test: *, between S1 and S2; **, between S2 and S3; ***, between S3 and S1 inside the same substrate concentration group. For the production of glutamine and glutamate found in the absence of exogenous substrate, see Table 1.

Segment...	Aspartate [0.05 mM] ($n = 6$)			Aspartate [5 mM] ($n = 9$)		
	S1 (12–24)	S2 (11–29)	S3 (6–27)	S1 (7–28)	S2 (5–32)	S3 (6–26)
Glutamine						
Mean	9.3	19.4	19.6	41.0	39.7	37.6
$\pm$ S.E.M.	1.4	4.2	3.7	3.7	3.2	4.2
$P < 0.05$	*		***			
Glutamate						
Mean	5.8	9.0	7.8	13.2	32.2	23.3
$\pm$ S.E.M.	0.9	1.4	1.1	1.8	5.1	2.9
$P < 0.05$				*		***
Glutamine plus glutamate						
Mean	15.1	28.4	27.4	54.2	71.9	60.9
$\pm$ S.E.M.	1.9	0.9	4.0	4.9	7.2	6.2
$P < 0.05$	*		***			

127% ($P < 0.05$), 257% ($P < 0.05$) and 198% ($P < 0.05$) in the S1, S2 and S3 segments, respectively. It is noteworthy that, with 5 mM aspartate as substrate, glutamate accumulation was high in the S2 and S3 segments, and that the glutamate accumulated by segment S2 was of a similar magnitude as the glutamine produced.

Comparison of the extent of glutamine synthesized/ glutamate accumulated (shown in Tables 1 and 2) from a physiological concentration of alanine with that from a physiological concentration of aspartate reveals that alanine (0.25 mM) was a better precursor of glutamine than aspartate (0.05 mM) in the S1 ($P < 0.05$), S2 ($P < 0.05$) and S3 ($P < 0.05$) segments; in contrast, glutamate accumulation from 0.25 mM alanine was not statistically different from that accumulated from 0.05 mM aspartate in the three tubular segments. With a 5 mM substrate concentration, glutamine production and glutamate accumulation were statistically higher from 5 mM alanine than from 5 mM aspartate only in the S2 segment ($P < 0.05$ and $P < 0.05$ respectively).

DISCUSSION

This study, which is the first in which glutamine synthesis was measured in freshly microdissected nephron segments, shows that the three segments (S1, S2 and S3) of the rabbit proximal tubule can synthesize glutamine from alanine and aspartate. In all segments, the linearity with both the time and extent of synthesis of glutamine from alanine and aspartate respectively indicates that these microdissected segments were metabolically viable. Indeed, glutamine synthesis is catalysed by glutamine synthetase, which is an ATP-dependent enzyme; in addition, this metabolic process requires co-ordination between the transport of alanine or aspartate and the operation of a certain number of enzymes (see Scheme 1), together with the functional integrity of mitochondria. In this respect, it is of interest to compare the rates of glutamine synthesis from 5 mM alanine and 5 mM aspartate, measured in suspensions of rabbit proximal tubules that con-

Scheme 1 Schematic representation of the pathway of glutamine synthesis from alanine and aspartate in rabbit kidney proximal tubule

This Scheme shows that two alanine molecules or two aspartate molecules are metabolized for each glutamate or glutamine molecule found. ALAT, alanine aminotransferase; ASPAT, aspartate aminotransferase; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; CS, citrate synthase; PEPCK, phosphoenol pyruvate carboxykinase; GLDH, glutamate dehydrogenase; GS, glutamine synthetase. Note that aconitate and isocitrate, two intermediates in the conversion of citrate into 2-oxoglutarate, as well as aconitase and isocitrate dehydrogenase, are omitted from this Scheme.

tained $7.3 \pm 0.4 \mu\text{mol}$ of ATP per g of tubular dry weight after 60 min of incubation, with those observed in the present study in the S1, S2 and S3 segments. The rates of glutamine synthesis from 5 mM alanine and 5 mM aspartate in suspensions of rabbit proximal tubules were 191 ± 13 and $130.9 \pm 14.4 \mu\text{mol} \cdot \text{g}^{-1}$ dry weight $\cdot \text{h}^{-1}$ respectively (means $\pm$ S.E.M., $n = 6$). Given that the dry weight of 1 mm of rabbit proximal tubule is $0.3 \mu\text{g}$ [21], it is easy to calculate that, on a dry-weight basis, the rates of glutamine synthesis from 5 mM alanine and 5 mM aspartate in our S1, S2 and S3 segments were of a similar magnitude as those measured in suspensions of rabbit proximal tubules. From these data, it seems justified to infer that our microdissected rabbit proximal tubular segments (S1, S2 and S3) contained an ATP concentration as high as that needed to support a high rate of glutamine synthesis in suspensions of rabbit proximal tubules.

Contrary to what one might have expected on the basis of enzyme activity measurements that showed a similar glutamine synthetase activity in all the segments of the rabbit proximal tubule [1], this study reveals that the capacity of the S1 segment to synthesize glutamine from a physiological concentration of both alanine and aspartate is about half that of the S2 and S3 segments (Tables 1 and 2). This illustrates the importance of studying the metabolic heterogeneity of the nephron, not only with intact renal cells, but also at physiological concentrations of substrates, a possibility offered by the microanalytical techniques used in the present study. In this respect, it is also important to note that, when 5 mM substrate concentration is used (as in most studies with suspensions of kidney tubules), the differences observed in the rates of glutamine synthesis among the S1, S2 and S3 segments are considerably diminished (see Tables 1 and 2, and the Results section). However, in the present study, such a high concentration of alanine and aspartate was particularly

useful to test the capacity of the three segments of the rabbit proximal tubule to increase their rate of glutamine synthesis.

On the basis of previous studies on glutamine synthesis from alanine, aspartate and glutamate in suspensions of isolated guinea-pig kidney tubules, or of isolated rabbit kidney tubules ([11,12,15,22–24]; our unpublished results), Scheme 1 shows how two alanines or two aspartates can be converted into one glutamate and/or one glutamine. Thus our results imply that all the enzymes needed for the conversion of alanine and aspartate into glutamine are active along the entire rabbit proximal tubule. In agreement with this is the observation that the activity of glutamine synthetase is present along the entire proximal tubule of the rabbit [1]. A high glutamate dehydrogenase activity has also been demonstrated along the rabbit proximal tubule [25,26]. Although the activity of the latter enzyme was found to be significantly lower in the pars recta than in the proximal convoluted tubule [25], this does not seem to be a rate-limiting factor in the synthesis of glutamine from physiological concentrations of alanine and aspartate in the S2 and S3 segments, which were found in the present study to synthesize more glutamine than the S1 segment (see Tables 1 and 2). High activities of phosphoenolpyruvate carboxykinase [27] and pyruvate kinase [28] have also been measured along the entire rabbit proximal tubule. To our knowledge, the activity of the other mitochondrial enzymes involved in the conversion of alanine or aspartate into glutamine in the present study (alanine or aspartate aminotransferase, pyruvate dehydrogenase, pyruvate carboxylase, citrate synthase, aconitase and isocitrate dehydrogenase) have never been measured along the rabbit proximal tubule. However, the high rate of glutamine synthesis found in the S1, S2 and S3 segments in the present study clearly indicates that numerous mitochondria are present, and that these mitochondrial enzymes are significantly active along the entire proximal tubule. In agreement with this view is the observation that succinate dehydrogenase has a high activity in the S1, S2 and S3 segments of the rabbit proximal tubule [25].

Since glutamine and to a lesser extent, glutamate, are almost the only non-volatile carbon end-products of alanine and aspartate metabolism in rabbit proximal tubules [11–13], one may determine, to a reasonable degree of accuracy, that the rates of alanine or aspartate utilization by the S1, S2 and S3 segments are twice the sum of the rates of utilization of glutamine and glutamate found (see Tables 1 and 2), because two alanine or two aspartate molecules are required for each glutamate or glutamine found. Given the high rates of alanine and aspartate utilization, which were of a similar magnitude as those of 5 mM alanine and 5 mM aspartate in a given segment of the proximal tubule, one may infer that high alanine/aspartate aminotransferase activities are present along the entire proximal tubule; in this respect, the proximal tubule of the rabbit differs from that of the rat, in which a high alanine aminotransferase activity was only observed in the pars recta [29]. The above calculations also strongly suggest that, in the presence of physiological concentrations of alanine and aspartate, the removal of these amino acids in the S1 segment was about half of that in the S2 and S3 segments. They also reveal that elevation of substrate concentration to 5 mM led to a large increase in substrate removal in the three segments, except for the S3 segment with alanine as substrate. For the latter experimental condition, alanine utilization remained lower in the S1 than in the S2 segment. These data indicate that each part of the proximal tubule has its own mechanism for adapting alanine or aspartate utilization when the level of these amino acids is increased. Whether this is due to different properties of the alanine and aspartate transport systems or to different activities of certain enzymes of alanine and aspartate metabolism in the

three segments of the proximal tubule is currently unknown, and merits further research. In this respect, it is interesting to compare the actual fluxes through glutamine synthetase measured in the present study with the activity of this enzyme measured in the S1, S2 and S3 segments by Burch et al [1]. The mean values for glutamine synthetase measured at 37 °C in the rabbit S1, S2 and S3 segments by this group were found to be 457, 516 and 383 mmol · kg⁻¹ dry weight · h⁻¹ respectively. Considering that the dry weight of 1 mm of rabbit proximal tubule is 0.3 µg, the mean glutamine synthetase activities in the rabbit S1, S2 and S3 segments are calculated to be 137, 155 and 115 pmol · mm⁻¹ · h⁻¹ respectively for a 1 mm strip of tubule. These values, which exceed by far the actual fluxes measured through glutamine synthetase in segments metabolizing 5 mM alanine or aspartate, indicate that this enzyme was not rate-limiting in our study.

The fact that, at physiological substrate concentration, glutamine synthesis was higher from alanine than from aspartate may simply be due to a higher rate of alanine removal, as suggested by the calculations made from the data shown in Tables 1 and 2. Such an argument cannot, however, be put forward to explain that glutamine synthesis was lower and glutamate accumulation higher in the S2 segment with 5 mM aspartate as substrate in comparison with 5 mM alanine as substrate; indeed, the data in Tables 1 and 2 suggest that alanine and aspartate removal were of a similar magnitude in this segment. Therefore a factor(s) specific for aspartate metabolism, which is difficult to identify from our results, limits the synthesis of glutamine in the S2 segment leading to more glutamate (and ammonia) accumulation.

It is interesting to note that, irrespective of the alanine or aspartate concentration that was employed in our study, glutamine, which is a molecule containing two nitrogen atoms, was not only the main carbon but also the main nitrogenous end-product of alanine and aspartate metabolism in all parts of the rabbit proximal tubules (see Tables 1 and 2). In this respect, it is important to note that ammonia was quantitatively a minor nitrogenous product of alanine and aspartate metabolism along the entire length of the proximal tubule at both low and high concentrations of alanine and aspartate; indeed, since the formation of one glutamate molecule, which contains only one nitrogen atom, requires the metabolism of two molecules of either alanine or aspartate and the action of glutamate dehydrogenase, one may deduce that the accumulation of one glutamate molecule was accompanied by the accumulation of one ammonia molecule. Ammonia accumulation has been found of a similar order of magnitude to that of glutamate accumulation in guinea-pig kidney tubules synthesizing glutamine from alanine or aspartate [22,23]. Thus one may infer that all parts of the rabbit proximal tubule seem to limit the release of ammonia. Further evidence for this may be obtained by calculating the nitrogen balance, i.e. the difference between the utilization of alanine or aspartate (one nitrogen for each alanine or aspartate utilized) and the sum of the accumulation of glutamate (one nitrogen for each glutamate found) and the formation of glutamine (two nitrogens for each glutamine found). Such calculations show that most of the nitrogen removed as alanine or aspartate by the S1, S2 and S3 segments was accounted for by the nitrogens recovered as glutamine, leaving little room for ammonia accumulation.

Physiological relevance

The fact that the entire rabbit proximal tubule has the capacity to synthesize glutamine from alanine and aspartate, whose removal from circulating blood by the rabbit kidney has been

demonstrated [30], underlies the potential physiological importance of renal glutamine synthesis in this species. Indeed, although this has not been tested experimentally to date, renal glutamine synthesis *in vivo* might possibly limit both the urinary excretion and release into the renal vein of the ammonia produced by the rabbit kidney. This would appear to be particularly important in the rabbit, a herbivorous species that forms an alkaline urine and does not need to synthesize and excrete many protons in the form of ammonium ions [2,6]. Given the neurotoxicity of high circulating levels of ammonia, renal glutamine synthesis in the rabbit might also appear to be a major detoxifying mechanism; indeed, without glutamine synthesis, most of the ammonia synthesized at high rates by the operation of glutamate dehydrogenase, which is very active in the rabbit proximal tubule [25,26] (as also revealed in the present study because one ammonia must be formed for each glutamine synthesized), would be released into the renal vein.

It is also interesting that virtually all the segments of the rabbit proximal tubule have the capacity to increase alanine and aspartate removal and glutamine synthesis. This might represent an important mechanism that allows the proximal tubule *in vivo* to buffer an increased renal amino acid load resulting from protein digestion, again limiting the renal release as ammonia of the alanine and aspartate amino nitrogen. The fact that, at physiological alanine and aspartate concentrations, the S1 segment synthesizes less glutamine than the S2 and S3 segments might be related to the primary physiological role played by the early part of the proximal tubule in reabsorption of most of the amino acids filtered through the glomerular barrier [31]. Finally, it is difficult to determine what the physiological advantage related to the saturation of glutamine synthesis might be, which occurs only in the S3 segment of the proximal tubule at a low physiological concentration of alanine, but not of aspartate.

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