Release and fixation of CO₂ by guinea-pig kidney tubules metabolizing aspartate

Guy MARTIN, Christian MICHOUDET, Nadine VINCENT and Gabriel BAVEREL*

Laboratoire de Physiologie Rénale et Métabolique, CNRS URA 1177 et INSERM U80, Faculté de Médecine Alexis Carrel, 69008 Lyon, France

1. The metabolism of L-[U-¹⁴C]aspartate, L-[1-¹⁴C]aspartate and L-[4-¹⁴C]aspartate was studied in isolated guinea-pig kidney tubules. 2. Oxidation of C-1 plus that of C-4 of aspartate accounted for 90–92 % of the CO₂ released from aspartate, whereas oxidation of the inner carbon atoms of aspartate (which occurs beyond the 2-oxoglutarate dehydrogenase step) represented only 8–10 % of aspartate carbon oxidation. 3. The formation of [1-¹⁴C]glutamine and [1-¹⁴C]glutamate from [1-¹⁴C]aspartate and [4-¹⁴C]aspartate indicated that about one-third of the oxaloacetate synthesized from aspartate underwent randomization at the level of fumarate. 4. With [U-¹⁴C]aspartate as substrate, the percentage of the C-1 of glutamate and glutamine found radiolabelled after 60 min of incubation was 92.7 % and 47.5 % in the absence and the presence of bicarbonate respectively. 5. That CO₂ fixation occurred at high rates in the presence of bicarbonate was demonstrated by incubating tubules with aspartate plus [¹⁴C]bicarbonate; under this condition, the label fixed was found in C-1 of glutamate, glutamine and aspartate, as well as in C-4 of aspartate, demonstrating not only randomization of aspartate carbon but also aspartate resynthesis secondary to oxaloacetate cycling via phosphoenolpyruvate carboxykinase, pyruvate kinase and pyruvate carboxylase. 6. The importance of CO₂ fixation in glutamine synthesis from aspartate is discussed in relation to the possible role of the guinea-pig kidney in systemic acid–base regulation *in vivo*.

INTRODUCTION

In a recent paper [1], we have shown that glutamine was the main carbon and nitrogenous product of aspartate metabolism in guinea-pig renal cortex. We have also presented experimental evidence for a pathway involving aspartate aminotransferase, glutamate dehydrogenase, glutamine synthetase, phosphoenolpyruvate carboxykinase, pyruvate kinase, pyruvate dehydrogenase and enzymes of the tricarboxylic acid cycle (citrate synthase, aconitase and isocitrate dehydrogenase) to explain the conversion of aspartate into glutamine [1]. According to this pathway, and in agreement with the observation that the amount of glutamine produced was about half that of aspartate removed [1], two molecules of aspartate were needed to form one molecule of glutamine.

In the same study [1], it was also shown that a significant fraction of the aspartate metabolized was oxidized to CO₂. However, many details of the metabolic fate of aspartate carbon in guinea-pig kidney tubules remained undefined. Thus it was uncertain to what extent each individual carbon atom of aspartate contributed to glutamine or CO₂, the main end-products of aspartate metabolism in this experimental model. In addition, we observed that guinea-pig kidney tubules incubated in the phosphate/saline medium used by Krebs [2,3] synthesized more glutamine from aspartate upon addition of bicarbonate, raising the question, so far unanswered, of whether bicarbonate acted as substrate or as regulator in this synthesis.

The present work was therefore undertaken to determine the relative contribution of each carbon atom of aspartate to CO₂ or glutamine, as well as the mechanism by which bicarbonate stimulates glutamine synthesis from aspartate. For this purpose, we incubated isolated guinea-pig kidney tubules with uniformly or specifically labelled [¹⁴C]aspartate, or with aspartate + [¹⁴C]bicarbonate, and determined the radioisotopic distribution in the carbon atoms of the major products.

The data obtained demonstrate that most of the CO₂ produced

was derived from C-1 and C-4 of aspartate and that complete oxidation of the aspartate carbon skeleton was very limited. In addition, this study reveals that aspartate metabolism in guinea-pig kidney tubules is much more complicated than previously thought and that, although it was not necessary for glutamine synthesis from aspartate, carboxylation of the pyruvate derived from aspartate also occurred at high rates, resulting in the labelling of aspartate, an observation indicative of the existence of aspartate resynthesis which involves aspartate aminotransferase, phosphoenolpyruvate carboxykinase, pyruvate kinase and pyruvate carboxylase.

MATERIALS AND METHODS

Animals

Guinea pigs (400–500 g) of the Dunkin–Hartley albino strain, body wt. 400–500 g (Iffa-Credo, St. Germain-sur-l'Arbresle, France), were fed on a standard pellet diet (U.A.R., Villemoisson-sur-Orge, France). Access to water was not limited.

Reagents

L-Aspartate, glutaminase (grade V), glutamate decarboxylase (grade V), acetazolamide and methionine sulphoximine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other enzymes and coenzymes were purchased from Boehringer (Meylan, France), and all other chemicals were reagent grade. L-[U-¹⁴C]Aspartic acid (200 mCi/mmol) and NaH¹⁴CO₃ (30–50 mCi/mmol) were from C.E.A. (Gif-sur-Yvette, France); L-[1-¹⁴C]aspartic acid (43.4 mCi/mmol) and L-[4-¹⁴C]aspartic acid (50 mCi/mmol) were supplied by New England Nuclear (Boston, MA, U.S.A.) and The Radiochemical Centre (Amersham, Bucks., U.K.) respectively.

Preparation of renal tubules and incubations

Isolated renal tubules were prepared from the kidneys of two

* To whom correspondence should be addressed.

Table 1. Metabolism of 5 mM-L-[U-¹⁴C]aspartate, -L-[4-¹⁴C]aspartate and -L-[1-¹⁴C]aspartate in guinea-pig kidney tubules incubated in Krebs–Henseleit medium

Kidney tubules (20.0 ± 1.3 mg dry wt. per flask) were incubated in Krebs–Henseleit medium as described in the Materials and methods section. Results (μmol/g dry wt.) for metabolite removal (–) or production are reported as means ± S.E.M. for five experiments. The paired Student's *t* test was used to measure the statistical difference between the production of ¹⁴CO₂ from [U-¹⁴C]aspartate against the sum of those from [1-¹⁴C]aspartate and [4-¹⁴C]aspartate, and that between the formation of [1-¹⁴C]glutamate + [1-¹⁴C]glutamine from [U-¹⁴C]aspartate against the sum of those from [1-¹⁴C]aspartate and [4-¹⁴C]aspartate; **P* < 0.01; ***P* < 0.001.

Experimental condition	Incubation time (min)	Metabolite removal or production						
		Aspartate	Ammonia	Alanine	Glutamate	Glutamine	¹⁴ CO ₂	[1- ¹⁴ C]Glu + [1- ¹⁴ C]Gln
5 mM-L-[U- ¹⁴ C]aspartate	30	–403.5 ± 20.3	57.2 ± 4.6	11.1 ± 1.7	41.1 ± 2.7	147.2 ± 9.7	690.0 ± 36.4*	90.9 ± 6.9
5 mM-L-[4- ¹⁴ C]aspartate	30	–392.1 ± 18.5	56.2 ± 4.0	10.8 ± 1.5	40.2 ± 1.9	142.1 ± 9.2	329.5 ± 16.0	55.8 ± 3.4
5 mM-L-[1- ¹⁴ C]aspartate	30	–392.7 ± 20.1	57.3 ± 3.5	10.1 ± 1.9	37.3 ± 2.4	136.2 ± 7.4	306.8 ± 13.7	27.7 ± 1.5
No substrate	30	1.1 ± 1.1	9.0 ± 1.3	–2.2 ± 0.5	–4.0 ± 0.4	21.7 ± 1.6	–	–
5 mM-L-[U- ¹⁴ C]aspartate	60	–756.1 ± 45.4	99.1 ± 11.1	10.2 ± 1.8	53.4 ± 5.6	301.9 ± 21.8	1296.1 ± 72.6**	168.7 ± 10.6**
5 mM-L-[4- ¹⁴ C]aspartate	60	–742.2 ± 40.8	99.6 ± 9.4	10.6 ± 1.6	54.3 ± 5.3	300.2 ± 20.8	578.7 ± 27.4	98.2 ± 6.3
5 mM-L-[1- ¹⁴ C]aspartate	60	–756.7 ± 35.2	99.4 ± 10.7	10.5 ± 1.7	52.5 ± 5.7	302.7 ± 18.9	583.2 ± 27.5	54.8 ± 3.0
No substrate	60	1.9 ± 1.2	31.1 ± 2.4	–0.8 ± 0.6	–2.2 ± 0.6	33.0 ± 1.9	–	–

guinea-pigs for each experiment, by the collagenase method described by Baverel *et al.* [4]. Incubations were performed for 30 or 60 min at 37 °C in a shaking water bath in 25 ml stoppered Erlenmeyer flasks, each with a centre well, in an atmosphere of O₂/CO₂ (19:1). Tubules were incubated in 4 ml of Krebs–Henseleit medium [5] containing either 5 mM-L-[¹⁴C]aspartate as substrate or 5 mM unlabelled L-aspartate in the presence of 25 mM-NaH¹⁴CO₃. In all experiments, each experimental condition was carried out in duplicate. Incubations were terminated by adding HClO₄ (final concn. 2%, v/v) to each flask. Then collection and measurement of the ¹⁴CO₂ released from [¹⁴C]aspartate or from [¹⁴C]bicarbonate were carried out as described by Baverel & Lund [6]. In all experiments, zero-time flasks, with and without substrates, were prepared by adding HClO₄ before the tubules. After removal of the denatured protein by centrifugation, the supernatant was neutralized with 20% (w/v) KOH for metabolite determination and measurement of bicarbonate fixation and of radioisotope distribution in non-volatile carbon products.

Analytical methods

Aspartate, alanine, glutamate, glutamine, ammonia and the dry weight of the amount of tubules added to the flasks were determined as described previously [4,7]. Formation of [1-¹⁴C]glutamine and [1-¹⁴C]glutamate was measured by use of the combined action of glutaminase and glutamate decarboxylase as described by Squires & Brosnan [8]. Fixation of ¹⁴CO₂ from [¹⁴C]bicarbonate was calculated by dividing the acid-stable radioactivity present in the incubation medium after incubation by the specific radioactivity of the total 'bicarbonate + CO₂' pool measured in the zero-time flasks.

The measurement of the radiolabelled bicarbonate carbon incorporation into C-1 and C-4 of aspartate was determined by combining enzymic transamination and chemical degradation by the following sequences of reactions.

(1) Transamination of aspartate with aspartate aminotransferase:

(2) Decarboxylation of oxaloacetate with Al₂(SO₄)₃, one of the most potent compounds known to accelerate oxaloacetate decarboxylation [9]:

(3) Decarboxylation of pyruvate with H₂O₂ [10]:

Reactions (1) and (2) were carried out in 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.5 M-2-oxoglutarate, 1% (w/v) Al₂(SO₄)₃ and labelled aspartate. The total volume was 2 ml in 25 ml Erlenmeyer flasks with a centre well. The reaction was then started by adding aspartate aminotransferase (9 units) to each flask. It was stopped after 6 h, a time after which the conversion of aspartate into pyruvate was complete, by adding HClO₄. Collection and measurement of the ¹⁴CO₂ released were performed as previously described [6]. Then, after a portion of the incubation medium had been transferred to another Erlenmeyer flask also equipped with a centre well, reaction (3) was carried out by adding H₂O₂ (1 M), and again the ¹⁴CO₂ released was collected and measured. By using commercially available L-[U-¹⁴C]aspartate, L-[1-¹⁴C]aspartate and L-[4-¹⁴C]aspartate, it was ascertained that all, and only, the radioactivity from the C-1 and C-4 of aspartate was released by this method.

Calculations

Net substrate utilization and product formation were cal-

culated as the difference between the total contents of the flask (tissue + medium) at the start (zero-time flasks) and after the period of incubation. The metabolic rates, reported as means $\pm$ S.E.M., are expressed as μ mol of substance removed or produced per g dry wt. of tubule fragment per unit time.

The rates of release of ¹⁴CO₂ from radioactive aspartate molecules were calculated by dividing the radioactivity in CO₂ by the specific radioactivity of the labelled aspartate carbon atoms determined in the zero-time samples for each experiment.

RESULTS AND DISCUSSION

Metabolism of [U-¹⁴C]aspartate, [1-¹⁴C]aspartate and [4-¹⁴C]aspartate in kidney tubules incubated in Krebs-Henseleit medium

As shown in Table 1, and in agreement with previous findings

Fig. 1. Fate of aspartate and bicarbonate carbon atoms during aspartate metabolism in guinea-pig kidney tubules

Aspartate is converted mainly into glutamine and CO₂ as described previously [1]. An asterisk denotes a radioactive carbon atom. C-1 of aspartate gives C-1 of oxaloacetate, which can give either C-1 of pyruvate and C-6 of isocitrate (which are subsequently released as CO₂) or C-4 of oxaloacetate (after randomization at the level of fumarate); C-4 of oxaloacetate can either be lost as CO₂ by the phosphoenolpyruvate carboxykinase and 2-oxoglutarate dehydrogenase reactions or give C-1 of glutamate and glutamine or C-4 of aspartate by aspartate resynthesis via the aspartate aminotransferase reaction. The fate of C-4 of aspartate can be found by following the same metabolic pathways. The ¹⁴CO₂ fixed from [¹⁴C]bicarbonate by pyruvate carboxylase gives C-4 of oxaloacetate. Abbreviations: ASP, aspartate; OAA, oxaloacetate; MAL, malate; FUM, fumarate; PEP, phosphoenolpyruvate; PYR, pyruvate; Ac-CoA, acetyl-CoA; CIT, citrate; ISOCIT, isocitrate; SUC, succinate; 2-OG, 2-oxoglutarate, which gives the carbon skeleton of glutamate (GLU) and then of glutamine (GLN). For simplicity, aconitate and succinyl-CoA are not shown in the figure.

[1], aspartate utilization, which, as expected, was not different when [U-¹⁴C]-, [4-¹⁴C]- or [1-¹⁴C]-aspartate was the substrate, was approximately linear with time over a 60 min incubation period. This was also true for the production of both glutamine and ¹⁴CO₂, the main products of aspartate metabolism in these tubules. Table 1 also shows that, after both 30 and 60 min of incubation, the release of ¹⁴CO₂ from [U-¹⁴C]aspartate was only slightly greater than the sum of the releases of ¹⁴CO₂ from [1-¹⁴C]aspartate and [4-¹⁴C]aspartate. This clearly indicates that C-2 and C-3 of aspartate contributed to the release of CO₂ only to a limited extent. Thus most (90–92 %) of the release of CO₂ from aspartate was due to partial oxidation of C-1 and C-4 of aspartate, and complete oxidation of the aspartate carbon skeleton removed was low.

Fig. 1 shows the metabolic routes used by isolated guinea-pig kidney tubules to synthesize one glutamine molecule from two aspartate molecules, as well as the enzymic steps at which the different carbon atoms of aspartate can be released as CO₂. It can be seen that C-1 and C-4, but not C-2 and C-3, of aspartate can be released as CO₂ before the 2-oxoglutarate dehydrogenase step; formation of CO₂ from C-4 of aspartate occurs via the phosphoenolpyruvate carboxykinase or the 2-oxoglutarate dehydrogenase reactions, whereas C-1 of aspartate can be released as CO₂ either at the pyruvate dehydrogenase or at the isocitrate dehydrogenase step.

The fact that the release of ¹⁴CO₂ from C-2 and C-3 of [U-¹⁴C]aspartate was low suggests that flux through 2-oxoglutarate dehydrogenase was also limited and that most of these inner carbon atoms of aspartate were removed from the tricarboxylic acid cycle and incorporated into glutamine before this enzymic step.

Fig. 1 also shows how [1-¹⁴C]glutamate and [1-¹⁴C]glutamine, which have the same carbon skeleton as 2-oxoglutarate, can be synthesized from [¹⁴C]aspartate via the formation of [4-¹⁴C]oxaloacetate and its subsequent conversion into citrate, aconitate, isocitrate and 2-oxoglutarate. The data of Table 1 reveal that [1-¹⁴C]glutamate + [1-¹⁴C]glutamine were formed not only from [U-¹⁴C]- and [4-¹⁴C]-aspartate but also from [1-¹⁴C]aspartate. Since C-1 of the glutamine and glutamate formed necessarily arises from C-4 of the oxaloacetate synthesized from aspartate, the latter observation means that part of the [1-¹⁴C]oxaloacetate formed by transamination of [1-¹⁴C]aspartate equilibrated with fumarate, a symmetrical molecule, via malate dehydrogenase and fumarase to give [4-¹⁴C]oxaloacetate; the percentage of the oxaloacetate molecules (formed by transamination of aspartate) undergoing such a randomization, calculated from the data of Table 1 with the following formula:

$$\frac{[1-^{14}\text{C}]\text{Glu} + [1-^{14}\text{C}]\text{Gln from } [1-^{14}\text{C}]\text{Asp}}{[1-^{14}\text{C}]\text{Glu} + [1-^{14}\text{C}]\text{Gln from } [1-^{14}\text{C}]\text{Asp and } [4-^{14}\text{C}]\text{Asp}} \times 100$$

was 33 % and 36 % after 30 and 60 min of incubation respectively.

The observation that the production of ¹⁴CO₂ from [U-¹⁴C]aspartate was only 8–10 % higher than the sum of the ¹⁴CO₂ productions from [1-¹⁴C]aspartate and [4-¹⁴C]aspartate indicates that only 8–10 % of the aspartate carbon skeleton passed through 2-oxoglutarate dehydrogenase in order to allow the release of ¹⁴CO₂ from the inner carbon atoms (C-2 and C-3) of aspartate, a process which can occur only beyond one turn of the tricarboxylic acid cycle (see Fig. 1). Further evidence that flux through 2-oxoglutarate dehydrogenase was very limited is provided by the finding that [1-¹⁴C]glutamate + [1-¹⁴C]glutamine synthesized from [U-¹⁴C]aspartate was only slightly but significantly ($P < 0.001$ after 60 min of incubation) higher than the sum of those synthesized from [1-¹⁴C]aspartate and [4-¹⁴C]-

Table 2. Metabolism of 5 mM-L-[U-¹⁴C]aspartate, -L-[4-¹⁴C]aspartate and -L-[1-¹⁴C]aspartate in guinea-pig kidney tubules incubated in a phosphate/saline medium

Kidney tubules (16.7 ± 0.5 mg dry wt. per flask) were incubated in a phosphate/saline medium as described in the Materials and methods section. Results (μmol/g dry wt.) for metabolite removal (–) or production are reported as means ± S.E.M. for four experiments performed in duplicate.

Experimental condition	Incubation time (min)	Metabolite removal or production						
		Aspartate	Ammonia	Alanine	Glutamate	Glutamine	¹⁴ CO ₂	[1- ¹⁴ C]Glu + [1- ¹⁴ C]Gln
5 mM-L-[U- ¹⁴ C]aspartate	60	–634.0 ± 9.0	115.0 ± 8.0	19.1 ± 0.9	50.2 ± 1.9	224.2 ± 8.2	932.4 ± 59.6	254.4 ± 4.4
5 mM-L-[4- ¹⁴ C]aspartate	60	–631.7 ± 19.6	111.3 ± 10.4	18.1 ± 0.7	50.4 ± 1.8	227.2 ± 5.6	409.1 ± 20.9	149.4 ± 6.1
5 mM-L-[1- ¹⁴ C]aspartate	60	–617.5 ± 20.9	108.9 ± 9.6	17.5 ± 0.7	48.1 ± 2.2	224.1 ± 3.6	440.4 ± 27.5	62.1 ± 3.7
No substrate	60	–	61.5 ± 11.3	1.3 ± 1.0	–1.4 ± 0.6	5.6 ± 0.8	–	–

Table 3. Effect of bicarbonate concentration on glutamine synthesis from aspartate in guinea-pig kidney tubules incubated in phosphate/saline buffer

Kidney tubules (24.4 ± 3.1 mg dry wt. per flask), previously washed three times and resuspended in phosphate/saline buffer, pH 7.4 [3], were incubated for 60 min in 4 ml of the same buffer in an atmosphere of pure O₂ in the presence of 5 mM-L-aspartate. When NaHCO₃ was added to the phosphate/saline buffer, NaCl concentration was decreased to keep the osmolality of the medium constant, and the buffer as well as the flasks were gassed with a mixture of O₂ and CO₂ in appropriate concentrations to maintain pH 7.4 under all conditions. Results (μmol/g dry wt.) for the metabolite removal (–) or production (+) are reported as means ± S.E.M. for four experiments. Statistical difference was measured by the paired Student's *t* test against the control with 25 mM-bicarbonate: **P* < 0.05; ***P* < 0.02; ****P* < 0.01.

Buffer system	Metabolite removal or production				
	Aspartate	Alanine	Ammonia	Glutamate	Glutamine
Phosphate/saline	–659.0	+11.4	+116.2	+48.5	+285.5
+ 25 mM-HCO ₃ [–] ; 5 % CO ₂ in O ₂	± 11.6	± 1.8	± 9.9	± 4.4	± 4.8
Phosphate/saline	–635.0	+12.6	+126.7	+44.4	+264.8
+ 10 mM-HCO ₃ [–] ; 2.1 % CO ₂ in O ₂	± 8.9**	± 1.8	± 11.5***	± 4.1*	± 6.7***
Phosphate/saline; pure O ₂	–573.6 ± 23.2***	+16.0 ± 2.4***	+128.0 ± 8.7***	+38.8 ± 1.5*	+231.4 ± 2.2***

aspartate, indicating that a small proportion of C-2 and C-3 of aspartate contributed to the formation of C-1 of glutamate and glutamine, a process which can occur only beyond the first turn of the tricarboxylic acid cycle (see Fig. 1).

The observation that only about half the C-1 of glutamine and glutamate was radiolabelled when [U-¹⁴C]aspartate was the substrate (Table 1) implies that carbon atoms other than those of aspartate contributed to C-1 of glutamine and glutamate. Nitrogen-balance calculations from the data given in Table 1 indicate that virtually all the nitrogen found as glutamine, glutamate, ammonia and alanine could be accounted for by the nitrogen removed as aspartate, which means that very little endogenous substrates contributed to the formation of the products found in these experiments (see Table 1). Therefore, with [U-¹⁴C]aspartate as substrate, the unlabelled C-1 of glutamine and glutamate must come from sources other than endogenous substrates. The fact that, in the presence of [U-¹⁴C]aspartate as substrate, the only other unlabelled source of carbon in the medium was bicarbonate led us to consider the possibility that CO₂ fixation occurred during aspartate metabolism by the action of pyruvate carboxylase, which is known to be present in guinea-pig renal cortex [11,12].

That indeed the presence of bicarbonate in the medium was responsible for the formation of unlabelled C-1 of glutamine and glutamate from [U-¹⁴C]aspartate (see Table 1) is demonstrated by the results shown in Table 2. When tubules were incubated in phosphate/saline medium with pure oxygen as gas phase and in the presence of [U-¹⁴C]aspartate as substrate, almost all the C-1

of the glutamine and glutamate synthesized was found to be radiolabelled (see Table 2). Similarly, the formation of [1-¹⁴C]glutamine + [1-¹⁴C]glutamate from either [1-¹⁴C]aspartate or [4-¹⁴C]aspartate was much higher when tubules were incubated in the absence of bicarbonate (Table 2) than in the presence of bicarbonate (Table 1), despite a lower rate of aspartate utilization in the absence of bicarbonate (see Tables 1 and 2). Addition of 25 mM-bicarbonate with an atmosphere of O₂/CO₂ (19:1) (Table 3) induced a 15 % stimulation of aspartate utilization with a concomitant 24 % increase in glutamine + glutamate synthesis by guinea-pig kidney tubules incubated for 60 min in phosphate/saline medium as described in Table 2. Therefore these results, which confirm that the presence of bicarbonate is not mandatory to observe the conversion of aspartate into glutamine by guinea-pig kidney tubules [1], strongly suggest that, in the presence of bicarbonate, CO₂ fixation occurred during such a conversion.

CO₂ fixation from [¹⁴C]bicarbonate during glutamine synthesis from aspartate: effect of acetazolamide

That CO₂ fixation occurred at high rates during aspartate metabolism was clearly demonstrated by incubating tubules in Krebs–Henseleit medium containing 25 mM-NaH¹⁴CO₃ (Table 4); under this condition (aspartate + [¹⁴C]bicarbonate), the rate of ¹⁴CO₂ fixation observed was in the same range as that of glutamine synthesis (Table 4). Contrary to our expectations, the formation of [1-¹⁴C]glutamine + [1-¹⁴C]glutamate could not fully account for the ¹⁴CO₂ fixed, and the fate of a significant fraction of the ¹⁴CO₂ fixed could not be determined in these experiments.

Kidney tubules (19.2 ± 1.1 mg dry wt. per flask) were incubated in Krebs-Henseleit medium containing $25 \text{ mM-NaH}^{14}\text{CO}_3$ as described in the Materials and methods section. Results ($\mu\text{mol/g dry wt.}$) for metabolite removal (—) or production are reported as means $\pm$ S.E.M. for four experiments performed in duplicate. $^{14}\text{CO}_2$ fixation and incorporation into glutamine and glutamate were measured as described in the Materials and methods section. The 'unexplained' $^{14}\text{CO}_2$ fixed was calculated as the difference between the total $^{14}\text{CO}_2$ fixation minus the $^{14}\text{CO}_2$ recovered as $[1-^{14}\text{C}]\text{glutamate} + [1-^{14}\text{C}]\text{glutamine}$. Statistical difference was measured by the paired Student's *t* test against the control without methionine sulphoximine (MSO): NS, not significant.

Experimental condition	Metabolite removal or production				¹⁴ CO ₂ fixed					
	Aspartate	Alanine	Ammonia	Glutamate	Glutamine	Total	[1- ¹⁴ C]Glu + [1- ¹⁴ C]Gln	[1- ¹⁴ C]Glu	[1- ¹⁴ C]Gln	Unexplained
5 mM-L-aspartate	-809.9	15.9	46.6	64.8	335.0	301.9	194.5	33.1	161.4	107.4
+ 25 mM-NaH ¹⁴ CO ₃	± 43.5	± 1.6	± 20.7	± 18.5	± 18.5	± 29.3	± 13.6	± 6.5	± 8.8	± 17.7
5 mM-L-aspartate	-627.5	46.7	397.8	231.8	-2.1	279.9	94.6	98.6	-4.0	184.4
+ 25 mM-NaH ¹⁴ CO ₃	± 29.0	± 3.9	± 36.8	± 10.4	± 5.2	± 49.9	± 9.0	± 9.1	± 5.6	± 46.0
+ 5 mM-MSO	<i>P</i> < 0.02	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001	NS	<i>P</i> < 0.01	<i>P</i> < 0.001	<i>P</i> < 0.001	NS
No substrate	4.0	-1.2	17.2	1.0	46.0	32.5	28.7	3.4	25.3	3.8
+ 25 mM-NaH ¹⁴ CO ₃	± 3.4	± 0.5	± 7.5	± 1.2	± 1.7	± 3.1	± 4.0	± 0.9	± 4.7	± 4.4
No substrate	7.7	1.1	150.2	13.4	-10.1	21.4	6.0	2.8	3.2	15.5
+ 25 mM-NaH ¹⁴ CO ₃	± 1.9	± 0.9	± 16.2	± 3.1	± 2.3	± 3.7	± 2.5	± 0.3	± 2.4	± 2.4
+ 5 mM-MSO	NS	NS	<i>P</i> < 0.001	<i>P</i> < 0.05	<i>P</i> < 0.001	<i>P</i> < 0.01	<i>P</i> < 0.02	NS	<i>P</i> < 0.05	NS

Addition of acetazolamide (0.5 and 1 mM), an inhibitor of carbonic anhydrase [14], did not alter the amount of $^{14}\text{CO}_2$ fixed from 25 mM- $\text{NaH}^{14}\text{CO}_3$ ($n = 3$; results not shown), suggesting that this enzyme does not catalyse a mandatory step in the provision of bicarbonate to the pyruvate carboxylase reaction. Although this finding seems at first sight surprising, it should be pointed out that guinea-pig kidney mitochondria lack carbonic anhydrase activity [15]. Therefore, in view of the poorly permeant nature of bicarbonate [16], these results strongly suggest that in our guinea-pig kidney tubules intramitochondrial alkalinity was adequate to convert sufficient amounts of CO_2 into bicarbonate for the operation of the pyruvate carboxylase reaction.

Comparing the data in Table 4 with those in Table 5, it may be surprising at first sight that the sum of [^{14}C]aspartate, [^{14}C]aspartate and 'unexplained $^{14}\text{CO}_2$ fixed' in Table 5 is about twice the 'unexplained $^{14}\text{CO}_2$ fixed' in Table 4. This can be explained by the fact that much more aspartate, and therefore much more $^{14}\text{CO}_2$, fixed as C-1 and C-4 of aspartate, were left at

Table 5. Fate of the $^{14}\text{CO}_2$ fixed during the metabolism of 5 mM-L-aspartate in guinea-pig kidney tubules

Kidney tubules (13.6 ± 1.5 mg dry wt. per flask) were incubated in Krebs-Henseleit medium containing 25 mM- $\text{NaH}^{14}\text{CO}_2$ as described in the Materials and methods section. Results ($\mu\text{mol/g dry wt.}$) for metabolite removal (-) or production are reported as means $\pm$ S.E.M. for four experiments performed in duplicate. $^{14}\text{CO}_2$ fixation and incorporation into glutamine, glutamate and aspartate were measured as described in the Materials and methods section. The 'unexplained' $^{14}\text{CO}_2$ fixed was calculated as the total $^{14}\text{CO}_2$ fixation minus the $^{14}\text{CO}_2$ found as C-1 of glutamine, glutamate and aspartate and as C-4 of aspartate.

Experimental condition	Incubation time (min)	Metabolite removal or production					$^{14}\text{CO}_2$				
		Aspartate	Alanine	Ammonia	Glutamate	Glutamine	Total	[1- ^{14}C]Glu + [1- ^{14}C]Gln	[4- ^{14}C]Asp	[1- ^{14}C]Asp	Unexplained
5 mM-L-aspartate + 25 mM- $\text{NaH}^{14}\text{CO}_3$	60	-741.0 $\pm$ 42.3	17.0 $\pm$ 1.8	78.7 $\pm$ 8.0	66.8 $\pm$ 7.0	333.6 $\pm$ 23.6	391.1 $\pm$ 16.8	194.0 $\pm$ 11.3	101.0 $\pm$ 7.7	51.3 $\pm$ 2.3	44.9 $\pm$ 8.9
No substrate + 25 mM- $\text{NaH}^{14}\text{CO}_3$	60	0.3 $\pm$ 1.8	0.5 $\pm$ 0.4	10.8 $\pm$ 9.3	-1.0 $\pm$ 0.6	34.5 $\pm$ 4.0	24.7 $\pm$ 1.7	19.0 $\pm$ 2.2	0.3 $\pm$ 0.1	2.0 $\pm$ 0.5	3.4 $\pm$ 1.7

the end of the incubation in the experiments whose results are presented in Table 5, because a smaller amount of tubules was used in these experiments than in those whose results are given in Table 4.

Labelling of aspartate in these experiments (Table 5) implies that, despite a net utilization of aspartate, a bidirectional flux occurred at the level of aspartate aminotransferase, a process which can be explained by the near-equilibrium state of this enzyme. Labelling of aspartate after $^{14}\text{CO}_2$ fixation from [^{14}C]bicarbonate also reveals the existence of a so-called futile cycle (with oxaloacetate as substrate and final product) involving phosphoenolpyruvate carboxykinase, pyruvate kinase and pyruvate carboxylase. Such a cycle, whose existence is compatible with the activities of phosphoenolpyruvate carboxykinase and pyruvate kinase measured in guinea-pig kidney cortex [7], has been previously observed in isolated perfused rat kidney metabolizing aspartate [17] and in rat renal cortex slices incubated with pyruvate as substrate [18].

Moreover, our data establish the existence of aspartate resynthesis by aspartate aminotransferase working in the backward direction, after prior metabolism of aspartate via aspartate aminotransferase, phosphoenolpyruvate carboxykinase, pyruvate kinase and pyruvate carboxylase.

The fact that C-1 of aspartate was labelled after $^{14}\text{CO}_2$ fixation from [^{14}C]bicarbonate (Table 5) provides additional evidence that in guinea-pig kidney tubules the [$4\text{-}^{14}\text{C}$]oxaloacetate formed as a result of the action of pyruvate carboxylase equilibrated with fumarate to give subsequently [$1\text{-}^{14}\text{C}$]oxaloacetate via fumarase and malate dehydrogenase, and then [$1\text{-}^{14}\text{C}$]aspartate via aspartate aminotransferase. Comparison of the amounts of [$1\text{-}^{14}\text{C}$]aspartate and [$4\text{-}^{14}\text{C}$]aspartate found (see Table 4) confirms the observation made in the present study, on the basis of the synthesis of [$1\text{-}^{14}\text{C}$]glutamine + [$1\text{-}^{14}\text{C}$]glutamate from [$1\text{-}^{14}\text{C}$]aspartate and [$4\text{-}^{14}\text{C}$]aspartate (see Table 1), that about one-third of the oxaloacetate arising from aspartate underwent randomization at the level of fumarate.

Physiological significance

This study, which demonstrates that, in the presence of a physiological concentration of bicarbonate, fixation of CO_2 occurred at high rates to contribute to the formation of C-1 of glutamine by guinea-pig kidney tubules metabolizing aspartate, is of great interest. In view of the fact that the guinea-pig kidney is known *in vivo* to release glutamine [7] and excrete little NH_4^+ in an alkaline urine [19], it is conceivable that in this species renal pyruvate carboxylase participates to some extent not only in the control of the amount of bicarbonate either eliminated by the kidney in the form of glutamine or excreted in urine, but also, via glutamine carbon synthesis, in the control of the amount of NH_4^+ ions available for excretion in urine. According to this view, a metabolic acidosis which is accompanied by a low blood bicarbonate concentration would lead to a decrease in renal glutamate + glutamine synthesis secondarily to a decreased operation of pyruvate carboxylase and therefore to an increased renal availability and excretion of NH_4^+ ions. In agreement with this suggestion is our observation that, in guinea-pig tubules incubated with 5 mM-aspartate, the bicarbonate-dependent glutamate + glutamine synthesis (which represented 19 % of total glutamine synthesis) was decreased by 39 % when the bicarbonate concentration was decreased from 25 to 10 mM (Table 3). Although the renal uptake of aspartate is low *in vivo* because of its low circulating level [20,21], aspartate might be made available to the renal cells in significant amounts as an intermediate in glucose synthesis from lactate, a major substrate of the kidney *in vivo* [22]. However, it should be stressed that the extent to which

renal aspartate metabolism *in vivo* involves pyruvate carboxylase is at present unknown.

Finally, the existence and the significance under situations *in vivo* of the aspartate resynthesis and randomization found *in vitro* in the present study also remain to be demonstrated.

REFERENCES

1. Baverel, G., Martin, G. & Michoudet, C. (1990) *Biochem. J.* **268**, 437–442
2. Krebs, H. A. (1935) *Biochem. J.* **29**, 1951–1969
3. Krebs, H. A. (1933) *Hoppe-Seyler's Z. Physiol. Chem.* **217**, 191–227
4. Baverel, G., Bonnard, M., d'Armagnac de Castanet, E. & Pellet, M. (1978) *Kidney Int.* **14**, 567–575
5. Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
6. Baverel, G. & Lund, P. (1979) *Biochem. J.* **184**, 599–606
7. Baverel, G., Genoux, C., Forissier, M. & Pellet, M. (1980) *Biochem. J.* **188**, 873–880
8. Squires, E. J. & Brosnan, J. T. (1978) *Anal. Biochem.* **84**, 473–478
9. Krebs, H. A. (1942) *Biochem. J.* **36**, 303–305
10. Odessey, R. & Goldberg, A. L. (1979) *Biochem. J.* **178**, 475–489
11. Jones, C. T. & Ashton, I. K. (1976) *Arch. Biochem. Biophys.* **174**, 506–522
12. Robinson, B. H. (1976) *FEBS Lett.* **65**, 319–321
13. Meister, A. (1968) *Adv. Enzymol.* **31**, 183–218
14. Maren, T. H. (1967) *Physiol. Rev.* **47**, 595–781
15. Dodgson, S. J., Forster, R. E., Storey, B. T. & Mela, L. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5562–5566
16. Vincent, S. H. & Silverman, D. N. (1982) *J. Biol. Chem.* **257**, 6850–6855
17. Scaduto, R. C. & Davis, E. J. (1986) *Biochem. J.* **237**, 691–698
18. Rognstad, R. & Katz, J. (1972) *J. Biol. Chem.* **247**, 6047–6054
19. Janicki, R. H. & Goldstein, L. (1969) *Am. J. Physiol.* **216**, 1107–1110
20. Fukuda, S. & Kopple, J. D. (1980) *Miner. Electrolyte Metab.* **3**, 237–247
21. Bergman, E. N., Kaufman, C. F., Wolff, J. E. & Williams, H. H. (1974) *Am. J. Physiol.* **226**, 833–837
22. Kaufman, C. F. & Bergman, E. N. (1974) *Am. J. Physiol.* **226**, 827–832

Received 27 June 1991/23 December 1991; accepted 14 January 1992