Hepatic zonation of the catabolism of arginine and ornithine in the perfused rat liver

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The metabolism of ^{14}C-labelled arginine and ornithine was studied in the isolated, nonrecirculating, perfused rat liver. The catabolism of these amino acids required ornithine aminotransferase since treatment of rats with gabaculine, an inhibitor of this enzyme, decreased substantially the production of $^{14}CO_2$ from the ^{14}C-labelled amino acids. In the liver, ornithine aminotransferase is restricted to a small population of hepatocytes proximal to the terminal hepatic vein [Kuo, F. C., Hwu, W. L., Valle, D. and Darnell Jr., J. E. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 9468–9472], i.e. the perivenous subpopulation of hepatocytes. Catabolism of arginine requires arginase to convert arginine to ornithine which can then be catabolized through ornithine aminotransferase. The presence of arginase activity in the perivenous hepatocytes was demonstrated by experiments in which livers were perfused with [^{14}C]arginine in both antegrade and retrograde directions. Identical rates of $^{14}CO_2$ production were obtained in these experiments, a result which could only occur if the process of arginine catabolism through ornithine aminotransferase can be carried out in its entirety in the perivenous cells.

INTRODUCTION

Recent advances in arginine metabolism, such as the discovery of the nitric oxide pathway [1] and the newly discovered agmatine pathway in brain [2], have stimulated interest in this amino acid. There is clear evidence that changes in the degradation of arginine rather than altered *de novo* synthesis maintain arginine homoeostasis in man [3,4] and rats [5], under conditions of dietary arginine deficiency. There is, however, still a paucity of data relating to the catabolism of this amino acid in specific organs within mammals.

In liver, arginine and ornithine play important roles in the urea cycle. Their participation within this cycle, however, does not constitute net catabolism. Neither the nitric oxide pathway [6], nor polyamine synthesis from ornithine [7], contributes significantly to the catabolism of these amino acids in normal rat livers. Figure 1 represents the major catabolic route for both ornithine and arginine, occurring through the enzyme ornithine aminotransferase (OAT; EC 2.6.1.13). In this scheme, arginine is converted to ornithine by arginase (EC 3.5.3.1). Ornithine is then transaminated to glutamic semialdehyde which equilibrates spontaneously with pyrroline-5-carboxylate (P5C). P5C may enter the Krebs cycle via 2-oxoglutarate production from glutamate in the reaction catalysed by glutamate dehydrogenase (EC 1.4.1.2). The importance of OAT to the catabolism of arginine and ornithine is clearly demonstrated in a naturally occurring deficiency of this enzyme in humans. In patients with the genetic disorder 'gyrate atrophy', OAT levels are substantially lower than those found in normal individuals [8] and, as a consequence, plasma ornithine concentrations are markedly elevated [9]. Restricting dietary intake of arginine can return ornithine levels back to the normal range [10]. Also, in experiments carried out in mice, knock-out of the OAT gene [11] or inhibition of the enzyme [12,13] leads to a substantial increase in ornithine concentrations in all tissues studied, suggesting a significant role for OAT in ornithine catabolism in these mammals.

It is now appreciated that, in the liver, metabolic processes may be restricted in their location to different portions of the 'hepatic acinus', a phenomenon known as 'metabolic zonation' (for reviews, see [14–16]). A good example of this 'zonation' is ammonia detoxification in mammalian liver [17]. Urea cycle enzymes are located periportally while glutamine synthetase (GS, EC 6.3.1.2) is found exclusively in the perivenous cells. Swick et al. [18] were the first to postulate that OAT was restricted to perivenous hepatocytes when they demonstrated that it was found in a discrete subpopulation of hepatic mitochondria. This was directly confirmed by Darnell and coworkers [19], using *in situ* mRNA hybridization, who showed that OAT and GS colocalized to the same subpopulation of hepatocytes, i.e. the perivenous hepatocyte subpopulation. Thus, ornithine catabolism through OAT should occur perivenously. Whether or not the catabolism of arginine, through OAT, is exclusively a perivenous process depends on the presence or absence of an arginase within these cells.

We examined this question with experiments in which we measured the rate of oxidative catabolism of arginine and ornithine in the isolated, nonrecirculating, perfused rat liver. We perfused alternately in either the antegrade or retrograde direction. Arginine catabolism in the retrograde direction requires an active arginase in the perivenous cells. We found that arginine catabolism was as rapid and complete in the retrograde direction as it was in the antegrade direction, thus showing that the entire pathway for arginine catabolism is present in perivenous cells.

EXPERIMENTAL

Animals and diet

Male Sprague–Dawley rats were purchased from Charles River Ltd (Montreal, P.Q., Canada) and given free access to water and standard Purina chow diet. The rats (240–350 g) were given purified diets for a period of 3–5 days prior to the experiment. The diet was a modified AIN 76 diet [20]. It contained the following ingredients (in g/100 g of diet); casein, 14.8; L-methionine, 0.15; cornstarch, 17.0; sucrose, 53.3; corn oil, 5; vitamin mix (AIN 76), 1.0; mineral mix (AIN 76), 3.5; Alphacel,

Abbreviations used: P5C, pyrroline-5-carboxylate; OAT, ornithine aminotransferase; GS, glutamine synthetase; MSO, methionine sulphoximine.

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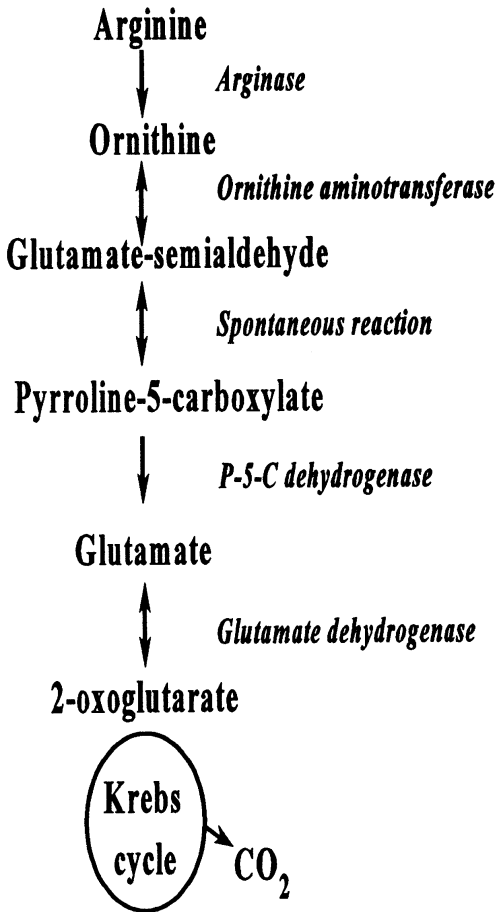

Figure 1 The pathway of catabolism of arginine and ornithine through ornithine aminotransferase

5; choline bitartrate, 0.2. Gabaculine (OAT inhibitor) was administered at a level of 50 mg/kg body by i.p. injection 2 h prior to the experiment. To inhibit GS, rats were given an i.p. injection of methionine sulphoximine (MSO) 2–2.5 h prior to the perfusion at a level of 50 mg/kg body weight.

Perfusion procedures

Nonrecirculating perfusions of rat livers were carried out as described by Sies [21]. Krebs–Henseleit medium (pH 7.4), gassed with O_2/CO_2 (19:1) with added lactate and pyruvate (2.1 and 0.3 mM, respectively), served as the basic perfusion medium. Amino acids, [U-^{14}C]arginine and [U-^{14}C]- or [1-^{14}C]-ornithine, were added at various concentrations at the times indicated in the results section. Oxygen consumption, perfusate pCO_2 and pH were monitored throughout the protocol by means of a blood-gas analyser (Model no. 238, Ciba Corning, Canada). Urea production was also measured [22]. For the antegrade/retrograde perfusions the flow direction was reversed from antegrade (porta-cava) to retrograde (cava-porta) during the perfusion experiment, as described by Häussinger [23]. For all perfusions, samples of the influent media were taken and effluent samples were collected at 5 min intervals. Samples for $^{14}CO_2$ analysis were taken under mineral oil. In the perfusions in which GS was inhibited by MSO injection (see Animals and diet), the perfusion medium also contained MSO at a concentration of 0.15 mM.

Measurement of $^{14}CO_2$ production

5 ml of each perfusate sample was injected into a stoppered 25 ml Erlenmeyer flask, containing 0.4 ml of 1 M HCl. The flasks were fitted with centre wells containing filter paper and 0.4 ml of NCS tissue solubilizer (Amersham Canada Ltd). The evolved CO_2 was trapped in the centre wells during incubation in a shaking water bath at 37 °C, for 1 h. The centre wells were transferred to scintillation vials containing 10 ml of scintillation fluid, and counted in a scintillation counter for ^{14}C. To account for any preformed $^{14}CO_2$ which might be present in the radioactive compounds, medium blanks were also prepared.

Enzyme assays and amino acid analysis

OAT was assayed as described by Herzfeld and Knox [24]. GS was assayed as previously described [25]. Protein concentration was determined using the Biuret method [26], after solubilization with deoxycholate, and using BSA as standard. Amino acids were analysed on a Beckman 121 MB amino acid analyser using a Benson D-X8, .25 Cation Xchange resin and a single column, 3 buffer lithium method [27].

Chemicals

[1-^{14}C]ornithine (specific activity 56 mCi/mmol) was purchased from Amersham Canada Ltd (Oakville, Ont., Canada). [U-^{14}C]arginine (specific activity 320 mCi/mmol) and [U-^{14}C]-ornithine (specific activity 257 mCi/mmol) were purchased from Dupont–NEN Canada Inc. (Mississauga, Ont., Canada). Diet components were purchased from ICN (Cleveland, OH, U.S.A.) except for L-methionine and cornstarch which were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Gabaculine (3-amino-2,3 dihydrobenzoate) and *o*-aminobenzaldehyde were also purchased from Sigma. All reagents used in the study were of analytical grade. Scintillation fluids were purchased from Fisher Scientific.

Presentation of data

The results are reported as means ± S.D. (with the exception of the saturation curve data). The kinetic data reported for the figures were determined using the GraphPad computer program (GraphPad Software, San Diego, CA, U.S.A.). Comparisons of data were made using Student's unpaired *t* test with $P < 0.05$ taken indicating a statistically significant difference.

RESULTS AND DISCUSSION

Characteristics of arginine and ornithine oxidation

To determine the kinetics of ornithine catabolism to CO_2, livers were perfused with varying concentrations of [1-^{14}C]ornithine. A typical experiment is shown in Figure 2A. These data fitted well to a rectangular hyperbola ($r^2 = 0.994$). V_{max} of CO_2 produced was calculated to be 63 nmol·min^{-1}·g^{-1} wet liver; the substrate concentration at which half-maximal ornithine oxidation is achieved is 2.4 mM. Figure 2B shows a typical substrate saturation curve for the production of CO_2, using [U-^{14}C]arginine as tracer. These data also fitted well to a rectangular hyperbola ($r^2 = 0.984$), and yielded a V_{max} of CO_2 produced of 210 nmol · min^{-1}·g^{-1} wet liver. Half-maximal arginine oxidation is achieved at an arginine concentration in the perfusion medium of 5.25 mM. It should be noted that the ornithine experiments were carried out with [1-^{14}C]ornithine and the arginine experiments with [U-^{14}C]arginine. The rate of arginine catabolism is not

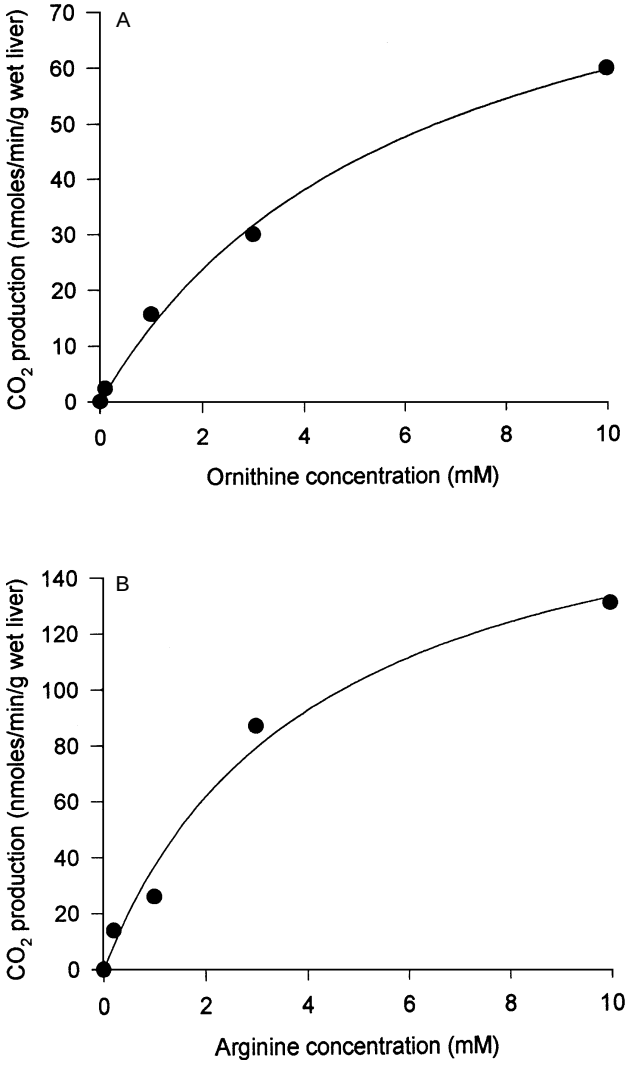

Figure 2 Substrate saturation curve for ornithine catabolism in the isolated, nonrecirculating, perfused rat liver

Livers were perfused in the antegrade direction with different concentrations of either (**A**) [1-^{14}C]ornithine or (**B**) [U-^{14}C]arginine as described in the Experimental section. Each panel represents data typical of two separate experiments.

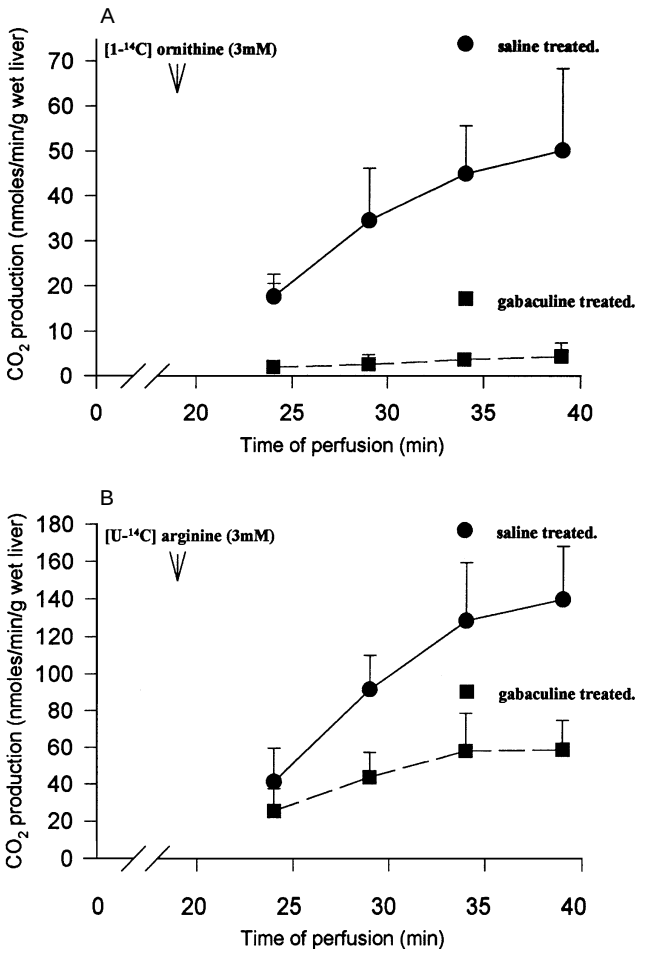

Figure 3 The effect of gabaculine on the catabolism of ornithine in the isolated, nonrecirculating, perfused rat liver

Gabaculine was administered intraperitoneally (50 mg/kg body weight) 2 h prior to perfusion. Control rats were administered saline. Livers were perfused in the antegrade direction with either (**A**) 3 mM [1-^{14}C]ornithine or (**B**) 3 mM [U-^{14}C]arginine as described in the Experimental section. Each point represents the mean $\pm$ S.D. for three independent experiments. Symbols: ●, livers from saline-treated rats; ■, livers from gabaculine-treated rats.

greater than the rate of ornithine catabolism; the higher V_{max} of the arginine data is due to the use of [U-^{14}C]arginine and the fact that there is a substantial oxidation of the carbon backbone (see below). These results demonstrate that the oxidative catabolism of arginine, and ornithine, in perfused rat livers is not saturated at physiological concentrations of these amino acids, since normal rat portal vein concentrations of ornithine and arginine are approximately 0.1 and 0.2 mM, respectively.

OAT is required for ornithine and arginine catabolism

Experiments were carried out to establish whether the production of CO_2 from ornithine occurred through OAT (as in Figure 1). Alonso and Rubio [13] have shown that gabaculine administration to mice at a level of 50 mg/kg body weight, causes a $> 90\%$ inhibition of mouse liver OAT. We found that this dose also markedly inhibited rat liver OAT. Figure 3A shows that the administration of gabaculine 2 h prior to the perfusion procedure resulted in a greater than 90% inhibition of CO_2 production using [1-^{14}C]ornithine as tracer. The means for CO_2 production, were 47.7 ± 14.4 and 4.1 ± 2.1 nmol $\cdot$ min$^{-1} \cdot$ g^{-1} wet liver, for saline-treated and gabaculine-treated animals, respectively (these were taken as the averages of the 34 and 39 min points). At the end of these perfusions a sample of liver was assayed for OAT. The OAT activity (2.4 ± 1.0 μmol $\cdot$ min$^{-1} \cdot$ g^{-1} protein in the control livers) was decreased by 80% (to 0.5 ± 0.1 μmol $\cdot$ min$^{-1} \cdot$ g^{-1} protein in livers from the gabaculine-treated rats). This catabolism of ornithine to CO_2, through OAT, in the perivenous cells implies that the enzymes P5C dehydrogenase also must be present in these cells (see pathway in Figure 1).

Gabaculine also inhibited arginine catabolism as shown in Figure 3B (CO_2 production of 134.2 ± 29.4 and 58.2 ± 29.4 nmol $\cdot$ min$^{-1} \cdot$ g^{-1} wet liver, for the means of the 34 and 39 min time points, for saline-treated and gabaculine-treated livers, respectively). Samples of liver taken at the end of the perfusions and assayed for OAT showed a 75% inhibition of this enzyme (4.1 ± 0.8 and 1.0 ± 0.5 μmol $\cdot$ min$^{-1} \cdot$ g^{-1} protein, in the control and gabaculine-treated livers, respectively). Thus arginine and

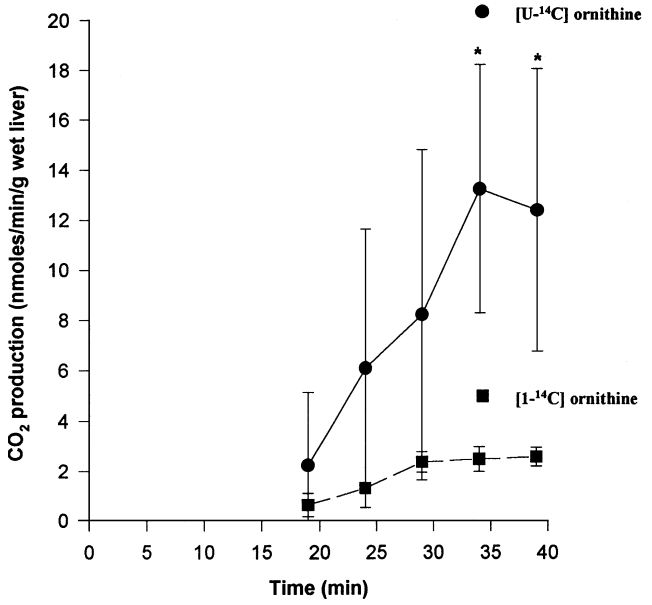

Figure 4 Comparison of $^{14}CO_2$ production from [U-^{14}C]ornithine to that from [1-^{14}C]ornithine

Livers were perfused in the antegrade direction with 0.1 mM [U-^{14}C]ornithine (●) or 0.1 mM [1-^{14}C]ornithine (■) as described in the Experimental section. The data represent the mean ± S.D. for three independent experiments. * denotes a significant difference at $P < 0.05$ from the [1-^{14}C]ornithine data.

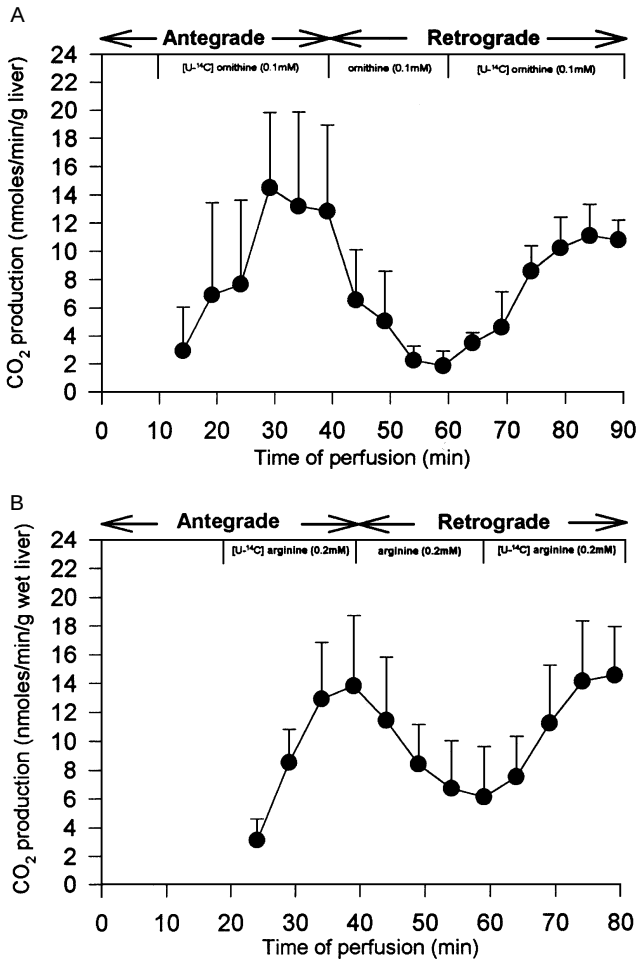

Figure 5 Ornithine and arginine catabolism during antegrade and retrograde perfusions of rat liver

Livers were perfused with 0.1 mM [U-^{14}C]ornithine (**A**), first in the antegrade direction (portal vein to vena cava) as described in the Experimental section. The direction of perfusion was reversed (retrograde) after 38 min; livers were perfused with 0.1 mM ornithine for 20 min to wash out ^{14}C intermediates and then 0.1 mM [U-^{14}C]ornithine was added to the perfusion medium. Similar experiments were carried out using 0.2 mM [U-^{14}C]arginine and these are represented in (**B**). There is no statistically significant difference between plateau CO_2 production during the antegrade perfusion and that during the retrograde perfusion in either set of experiments.

ornithine catabolism occur through OAT as is evident from the substantial inhibition by gabaculine. There was a higher residual activity of OAT in the gabaculine-treated rats that were perfused with arginine compared with those perfused with ornithine (1.0 ± 0.5 vs. 0.5 ± 0.1 μmol·min^{-1}·g^{-1} protein). This largely accounts for the observation that gabaculine-inhibited CO_2 production from arginine is less than CO_2 production from ornithine. Any residual CO_2 production, from labelled precursors, subsequent to treatment with gabaculine is attributable, in part, to the remaining OAT activity and to a very minor degree to pathways of arginine and ornithine catabolism which do not require OAT, e.g. the reaction catalysed by ornithine decarboxylase (EC 4.1.1.17), which is not inhibited by gabaculine [28,29] and by the action of arginine decarboxylase in agmatine biosynthesis [2].

Fate of the carbon skeleton of ornithine

When [1-^{14}C]ornithine is catabolized to yield $^{14}CO_2$, this CO_2 is released from the carbon at position 1 in the Krebs cycle by the enzyme 2-oxoglutarate dehydrogenase (EC 1.2.4.2). This gives us no information, however, as to the fate of the other four carbon atoms. We, therefore, compared the rate of CO_2 production, in isolated perfused rat livers, using [U-^{14}C]ornithine (a tracer for carbons 1–5) to that using [1-^{14}C]ornithine (a tracer for the CO_2 production from position 1), both at the physiological concentration of 0.1 mM (Figure 4). A plateau in CO_2 production was reached by 34 min, so an average of the 34 and 39 min samples were used to calculate CO_2 production. CO_2 production from positions 1–5 of ornithine was ~ 13 nmol·min^{-1}·g^{-1} wet liver (using [U-^{14}C]ornithine as tracer), almost five times the rate, 2.75 nmol·min^{-1}·g^{-1} wet liver, produced from the carbon at position 1 of ornithine. Thus the ornithine carbon that enters the Krebs cycle is substantially oxidized. Entry of ornithine carbon (as 2-oxoglutarate) into the Krebs cycle does not, of itself, result in its oxidation; rather it would just increase the total pool of Krebs cycle intermediates. Complete oxidation of the ornithine requires removal of intermediates from the Krebs cycle and their conversion to pyruvate which may be completely oxidized. This can be accomplished by malic enzyme (EC 1.1.1.39) (which converts malate to pyruvate) or phosphoenolpyruvate carboxykinase (EC 4.1.1.49) [which converts oxaloacetate to phosphoenolpyruvate, which is then readily converted to pyruvate by pyruvate kinase (EC 2.7.1.40)]. Thus perivenous cells, the exclusive location of hepatic OAT, must also contain either the malic enzyme or phosphoenolpyruvate carboxykinase. Phosphoenolpyruvate carboxykinase mRNA is relatively poorly expressed in perivenous hepatocytes [30] so malic enzyme may be the more likely candidate, but this needs to be addressed experimentally.

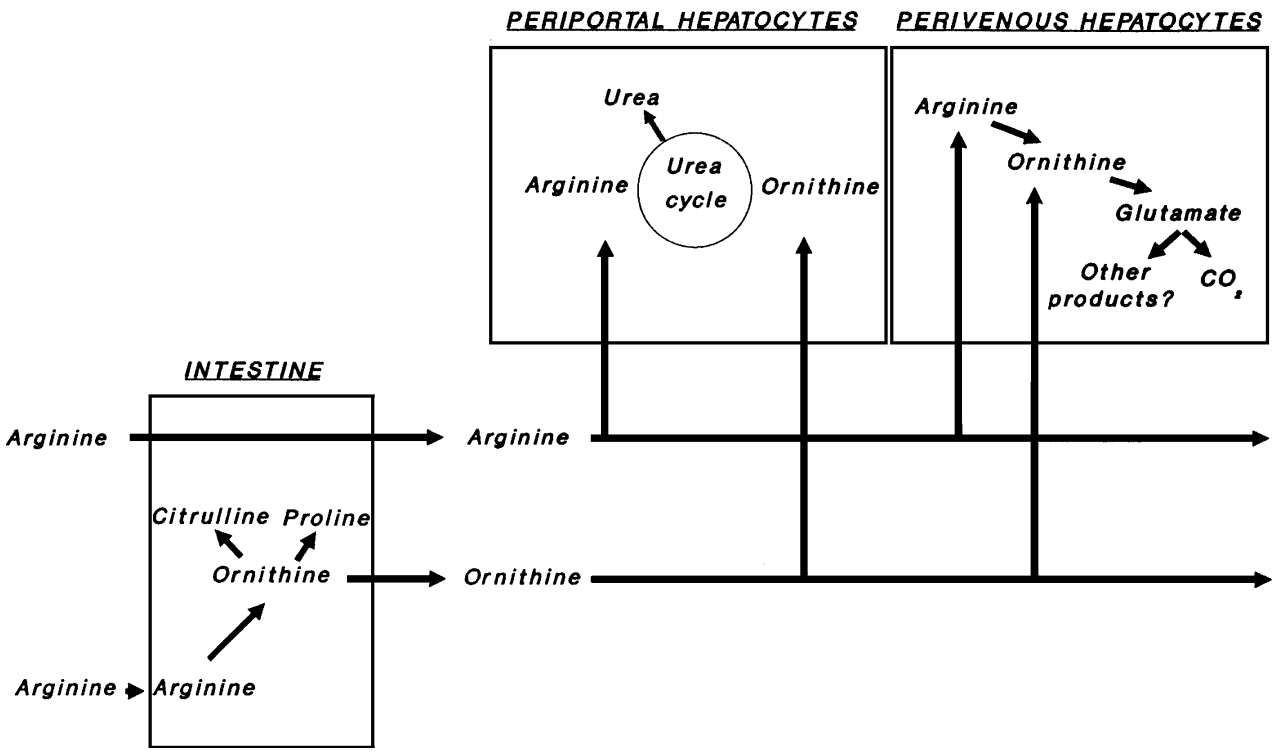

Figure 6 Proposed pathway for arginine metabolism in the splanchnic region

Dietary arginine, a portion of which is converted to ornithine in the intestine, may replete the urea cycle with intermediates in the periportal hepatocytes, with only the excess becoming available for catabolism in the perivenous hepatocytes.

Antegrade and retrograde perfusions

Comparison of perfusion in antegrade and retrograde directions may be used to investigate the zonation of hepatic metabolism, as exemplified by the elegant work of Häussinger [23] on hepatic nitrogen metabolism. Thus, to determine whether the entire process of arginine catabolism can occur in the perivenous cells, a series of antegrade/retrograde perfusions was carried out. A perivenously located arginase is required in order for the perfused liver to catabolize arginine with equal efficiency in antegrade or retrograde directions. This follows from the restricted localization of OAT to the perivenous region. However, if arginine must be hydrolysed to ornithine by arginase in the periportal hepatocytes we would expect no arginine catabolism when livers are perfused in the retrograde direction. Figure 5A shows that the direction of perfusion did not affect CO_2 production from ornithine, using [U-^{14}C]ornithine. Neither did the change in perfusion direction affect the flow rate, oxygen consumption or urea production of these livers (results not shown). We, therefore, proceeded to examine the effect of perfusion direction on arginine metabolism. Figure 5B clearly shows that the production of CO_2 from [U-^{14}C]arginine is the same when livers are perfused in antegrade or retrograde directions. These experiments were repeated in rats in which GS was inhibited ($\sim 95\%$) by treatment with MSO (details are provided in the Experimental section). This treatment had no effect on the rates of CO_2 production either in the antegrade or retrograde directions. These results indicate that the perivenous cells must contain an arginase. A similar conclusion may be drawn from the relative rates of arginine and ornithine catabolism. For example, the data in Figures 5A and 5B show that comparable rates of CO_2 production by livers (~ 13 nmol$\cdot$min$^{-1}\cdot$g^{-1} liver) were obtained with either 0.1 mM [U-^{14}C]ornithine or 0.2 mM [U-^{14}C]arginine. If the catabolism of arginine involved the action of a periportal arginase, or indeed an arginase contained within non-parenchymal cells, which could deliver ornithine to a perivenous OAT then about half of the arginine would have to be metabolized in the periportal region to deliver sufficient ornithine to the perivenous cells. However, we find that the arginine concentration leaving the liver is $> 85\%$ of that entering it, i.e. only a small fraction is catabolized.

It is well known that rat liver contains a potent arginase in the cytoplasm of the periportal hepatocytes that functions in the urea cycle. This is known as the A1 isoenzyme of arginase (also known as 'liver type'). However, Cheung and Raijman [31] have reported the presence of a small percentage of arginase, in the rat liver, which is closely associated with the mitochondrial fraction. Furthermore, this mitochondrially associated arginase has the same Mn^{2+} cofactor requirements as does the kidney type arginase, or the A2 isoenzyme [32]. In human liver, the vast majority of the arginase is A1 but there is also a small percentage (2%) of A2 [33]. In the kidney, this isoenzyme is co-expressed in the same cells (proximal straight tubules) as OAT [34] and, in addition, both are found in the mitochondria. It is quite conceivable that this mitochondrially associated hepatic arginase may occur in the perivenous hepatocytes where it participates in arginine catabolism. However, identification of the precise perivenous arginase requires further work.

Our current view of the zonation of hepatic arginine metabolism is shown in Figure 6. This shows both arginine and ornithine absorption into the hepatic portal vein since it is known that about one third of dietary arginine is hydrolysed by intestinal arginase with the release of ornithine [35]. Thereafter,

they may be taken up into periportal hepatocytes but only in small amounts since they are not catabolized there but may replenish the urea cycle of any depleted cycle intermediates. Both ornithine and arginine are taken up and oxidized to CO_2 in perivenous hepatocytes. This means that the entire catabolic pathway must exist in these cells. It is possible that some arginine and ornithine may be converted to other products, such as glutamine as postulated by Darnell et al. [19], since OAT is co-expressed in the same perivenous cells as glutamine synthetase. The extent to which this occurs remains to be determined. Finally, we may speculate on the function of separate zonation of the two major pathways of hepatic arginine catabolism. This arrangement ensures that the urea cycle has the opportunity to be replenished with cycle intermediates before these become available for catabolism, thus giving primacy to the vital function of ammonia detoxification.

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