Participation of ornithine aminotransferase in the synthesis and catabolism of ornithine in mice

Studies using gabaculine and arginine deprivation

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Gabaculine, a potent suicide inhibitor of ornithine aminotransferase (OAT), at a dose of 50 mg/kg inhibited this enzyme in mouse tissues and dramatically increased tissue ornithine concentrations, whether or not arginine was present in the diet. Thus even under arginine deprivation there is catabolism of ornithine which involves OAT. This was confirmed by administration of [^{14}C]ornithine to arginine-deprived mice. Gabaculine (3-amino-2,3-dihydrobenzoic acid) drastically decreased the release of $^{14}\text{CO}_2$ and increased the radioactivity in the basic amino acids in the tissues. When [^{14}C]glutamate was injected into mice deprived of arginine, a significant amount of radioactivity was recovered in tissue ornithine and arginine, and gabaculine decreased this labelling by about two-thirds, indicating that ornithine was synthesized *in vivo* from glutamate via OAT. In addition, we failed to detect in liver and small intestine α -N-acetylornithine, N-acetylglutamate kinase or N-acetylornithine aminotransferase, which are obligatory components of a potential route of ornithine synthesis from N-acetylglutamate. Our results indicate that at least 45 μmol of ornithine was synthesized and catabolized daily via OAT in the mouse deprived of arginine.

INTRODUCTION

In mammals, arginine is synthesized *de novo* [1–3] from glutamate via ornithine, which is then converted into arginine by enzymes of the urea cycle. In micro-organisms [4,5] and plants [6,7] ornithine is synthesized by the route (1) glutamate $\rightarrow$ N-acetylglutamate $\rightarrow$ N-acetylglutamyl γ -phosphate $\rightarrow$ N-acetylglutamate γ -semialdehyde $\rightarrow$ α -N-acetylornithine $\rightarrow$ ornithine, but in mammals it is generally believed that the route of synthesis is (2) glutamate $\rightarrow$ glutamic γ -semialdehyde $\rightarrow$ ornithine. In agreement with this view, Δ^1 -pyrroline-5-carboxylate (a cyclic compound formed spontaneously from glutamic γ -semialdehyde [8]) and ornithine were synthesized from glutamate in homogenates and mitochondrial fractions from rat intestinal mucosa [9–12], and addition of exogenous ornithine δ -aminotransferase (OAT; EC 2.6.1.13), the enzyme that catalyses the second step in sequence (2), increased the synthesis of ornithine [12]. Nevertheless, the reaction catalysed by OAT is reversible [8], and thus its implication in ornithine synthesis *in vitro* does not necessarily prove its implication *in vivo*. In fact, the apparent equilibrium favours semialdehyde synthesis [8], as is reflected in the accumulation of very high concentrations of ornithine in patients deficient in OAT [13].

In addition, acetylglutamate, the first intermediate in the pathway of synthesis of ornithine in micro-organisms and plants, is synthesized in the liver and intestine of mammals [14], where it activates carbamoyl-phosphate synthetase [15]. However, high concentrations of acetylglutamate were reported [16] in foetal rat liver, bearing no relation to the development of carbamoyl-phosphate synthetase. In isolated hepatocytes ornithine

concentrations paralleled those of acetylglutamate when glutamine was the ureagenic precursor [17], as might be expected if acetylglutamate were an intermediate in the pathway to ornithine.

To ascertain whether or not ornithine is synthesized in mammals by pathway (2), thus involving OAT, we tested the effects of the irreversible inhibition of this enzyme with gabaculine (3-amino-2,3-dihydrobenzoic acid) [18] on the concentrations and synthesis of ornithine in the mouse deprived of arginine. This species was used because it has no apparent arginine requirement for growth [19]. Our results show that the catabolism and oxidation of ornithine do not stop upon arginine deprivation and that OAT is involved both in the synthesis and in the catabolism of ornithine. As an additional confirmation that pathway (1) is not implicated in ornithine synthesis in the mouse, we searched, with negative results, for acetylornithine, acetylglutamate kinase (EC 2.7.2.8) and acetylornithine δ -aminotransferase (EC 2.6.1.11), which are obligatory components of this potential route.

EXPERIMENTAL

Animals and diets

Swiss male albino mice (25–30 g body wt.) were fed on standard chow or a diet ([20]; from ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.) in which the protein is replaced by a mixture of 18 amino acids including or not arginine (designated here Arg⁺ and Arg⁻ respectively). When omitted, arginine was replaced by alanine. Food access was restricted to the period 17:00–08:00 h. Weight increase was decreased only by about one-third and one-fifth in weeks 2 and 3 respectively of arginine deprivation, but not afterwards.

Abbreviations used: OAT, ornithine δ -aminotransferase; Arg⁺ and Arg⁻ diets, amino acid diets with and without arginine.

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Chemicals

Gabaculine was from Fluka, and AG 50W-X8 (200–400 mesh, H⁺ form) from Bio-Rad. L-[1-¹⁴C]-Glutamate (55.3 Ci/mol), L-[1-¹⁴C]ornithine (55 Ci/mol) and L-[U-¹⁴C]ornithine (279 Ci/mol) were from New England Nuclear. [¹⁴C]Ornithine was purified further by using AG 50W [21]. α -N-Acetyl-L-[U-¹⁴C]-ornithine (279 Ci/mol), synthesized with glutamate acetyltransferase from *Pseudomonas aeruginosa* (PA01 strain) [22] by using 40 mM-acetylglutamate and 25 μ M-[U-¹⁴C]ornithine, was isolated by adsorption on a 0.1 ml AG 50W column, washing with 0.2 ml of 10 mM-HCl and elution with 1 ml of 10% (v/v) pyridine. [¹⁴C]-Carbamoyl phosphate (50 Ci/mol), synthesized with rat liver carbamoyl-phosphate synthetase [22] from 1 mM-NaH¹⁴CO₃ (50 Ci/mol; from Amersham), was separated from unchanged bicarbonate by bubbling with CO₂ at 0 °C and was stored at -70 °C at pH 7.

Enzyme assays

Mice were decapitated, and the small intestine (washed by flushing with cold saline), liver, kidneys, brain and about 0.5 g of muscle from the hindlegs and back were chilled, rinsed in saline, and homogenized at 0 °C with an Ultra-Turrax homogenizer, with 4 vol. of a solution of 0.14 M-KCl, 20 mM-potassium phosphate, pH 7.4, 4 μ g of pyridoxal phosphate/ml and 0.25% Triton X-100. OAT was assayed as described by Herzfeld & Knox [23]. A unit of enzyme synthesizes 1 μ mol of pyrroline-5-carboxylate/h. Acetylornithine δ -aminotransferase was assayed [24] in homogenates prepared as above, in mitochondrial fractions from liver [25] and small intestine [12], and in extracts [22] of *Ps. aeruginosa*. Acetylglutamate kinase was assayed [26] in the mitochondrial fractions from liver and intestinal mucosa. Mitochondria were either subjected to eight cycles of freezing and thawing, or treated with 0.1% Triton X-100. Ornithine transcarbamoylase was assayed in 1:20 (w/v) liver/water homogenates [27]; when the influence of gabaculine in the assay was tested, the concentration of ornithine was decreased 10-fold, to 0.25 mM.

Determination of ornithine and arginine

Mice were decapitated, and the brain, liver, kidneys, samples of blood from the neck and samples of muscle from the legs were homogenized within 0.5, 1, 2, 4 and 4 min, respectively, in 5 vol. of 1 M-HClO₄ at 0 °C with an Ultra-Turrax or a Potter (for brain) homogenizer. After incubation at 37 °C for 15 min of 20 μ l of the deproteinized and neutralized (KOH) supernatants with 30 μ l of 0.2 M-triethanolamine, pH 7.7, containing 0.6 unit of ornithine transcarbamylase (Sigma) and 1.5–20 nmol of [¹⁴C]carbamoyl phosphate (3000–45000 c.p.m./nmol), the mixture was acidified with 0.5 ml of 0.5 M-HClO₄ containing 2.5 mM-citrulline, heated for 45 min at 100 °C, and the radioactivity, corresponding to the citrulline generated from ornithine, was measured. Arginine was determined by difference in parallel assays with and without 5 μ g of arginase (Boehringer). Ornithine or arginine added to water or to tissue extracts yielded identical results, after subtraction of the endogenous content of the extracts. Results were linear up to 10 nmol of ornithine or arginine.

Determination of acetylornithine and acetylglutamate

The liver was freeze-clamped, and the small intestine

was flushed with cold saline and freeze-clamped. Acetylglutamate was assayed as in [28]. Acetylornithine in 0.5 ml of the extract prepared as in [28] was partially purified by using AG 50W (see under 'Chemicals'). The dried 10%-pyridine eluate, dissolved in water, was subjected to two-dimensional t.l.c. on 20 cm $\times$ 20 cm cellulose plates from Merck; solvents: first dimension, butan-1-ol/acetone/acetic acid/water (7:7:2:4, by vol.); second dimension, phenol/water (3:1, v/v). The rectangular area between R_f 0.29–0.41 (first dimension) and 0.73–0.81 (second dimension) was scraped off, packed in a Pasteur pipette and treated with 1 ml of water, and the fluid was analysed by h.p.l.c. [29]. Acetylornithine yielded a separate peak between tyrosine and methionine. Recovery, determined with acetyl[¹⁴C]-ornithine, was 75–80%. The method was validated by using *Escherichia coli* (a bacterium that synthesizes ornithine via acetylornithine [4]). We found 5 nmol of acetylornithine/g of *E. coli* cells at the mid-exponential phase of growth in minimal medium.

Incorporation of radioactivity from L-[1-¹⁴C]glutamate into arginine and ornithine

Two groups of four mice fed on the Arg⁻ diet for 2 months received intraperitoneally at 10:00 h either 0.25 ml of saline or 50 mg of gabaculine/kg in 0.25 ml of saline, and 1.5 h later 5 μ Ci of L-[1-¹⁴C]glutamate (100 nmol); 2 h later they were killed, the intestinal contents were washed out and the carcasses were acid-digested, freed from acid and clarified [1]. A 250 ml portion of the clear water solutions (12.5 ml/g body wt.) was fractionated on 1.5 cm $\times$ 18 cm columns of AG 50W [21], and the fractions were analysed for radioactivity and amino acid composition (by h.p.l.c. [29]). No amino acids were recovered in the effluent from the sample or from a 150 ml water washing. Neutral and acidic amino acids were then eluted with 300 ml of 10% pyridine; lysine, ornithine and about 20% of the arginine were eluted with 700 ml of 0.2 M-pyridine acetate, pH 5.1; and the remainder of the arginine was eluted alone, with 600 ml of 0.6 M-pyridine acetate, pH 5.1 (concentrations refer to pyridine).

RESULTS

Inhibition of OAT *in vivo* by gabaculine

Replacement of standard laboratory chow by the Arg⁺ or Arg⁻ diet had no effect on OAT activity [mean $\pm$ S.D. (units), for the entire organ] in the small intestine (315 $\pm$ 56), kidney (25 $\pm$ 3), brain (6.5 $\pm$ 1.9) or muscular tissue (128 $\pm$ 13). In agreement with [30], the activity in liver (32 $\pm$ 9) increased by about 25% with deprivation of arginine. Administration of $\geq$ 10 mg of gabaculine/kg inhibited OAT in < 1 h by > 90% in these organs, except in brain, where the dose required was 30-fold higher. At the dose chosen for subsequent studies (50 mg/kg) inhibition was persistent (< 15% of the activity recovered in 9 h) and complete in all organs except in brain; the residual activity in brain was negligible when compared with the total activity initially present in the whole animal.

Influence of gabaculine on other enzymes directly involved with ornithine

Acetylornithine δ -aminotransferase was not detected in the mouse (see below). In extracts of *Ps. aeruginosa*

Fig. 1. Influence of time, type of diet and gabaculine administration on ornithine concentrations in several tissues of the mouse

Mice were fed for 1 week with Arg⁺ or Arg⁻ diet, as indicated. After food removal at 08:00 on day 8, groups of three mice were killed at the indicated times and ornithine was determined. Arrows indicate the time (11:00 h) of intraperitoneal administration (8 ml/kg body wt.) of saline alone (○) or containing 50 mg of gabaculine/kg (●). Results are means $\pm$ S.D.

this enzyme was inhibited by only 20 and 40% by incubation (37 °C) with 1 mM- and 10 mM-gabaculine respectively. Thus gabaculine is a poor inhibitor of this enzyme. In contrast, bacterial [31] and rat liver [18] OAT were inhibited by 97 and 90%, respectively, by 10–12 μ M-gabaculine, and a 10 min incubation of rat hepatocytes with 50 μ M-gabaculine [32] totally inactivated OAT.

Intraperitoneal administration of 350 mg of gabaculine/kg or addition of 1.25 mM-gabaculine to the assay containing 0.25 mM-ornithine failed to inhibit liver

ornithine transcarbamylase. Ornithine decarboxylase was conclusively shown by others [18,33] not to be inhibited by gabaculine.

Influence of gabaculine administration on ornithine concentrations

Administration of 50 mg of gabaculine/kg to mice fed on either the Arg⁺ or Arg⁻ diet markedly increased ornithine in all organs studied (Fig. 1). The simplest explanation for this increase is that, even in mice deprived of arginine, ornithine was being catabolized via OAT

Fig. 2. Influence of time, type of diet and gabaculine administration on the total amounts of ornithine and arginine in the entire organs and tissues analysed (liver, muscle, kidney, brain and blood)

Results, which are the means for groups of three mice, were calculated from the concentrations of ornithine and arginine in the tissues of the mice in Fig. 1 and from the experimental weights of liver, brain and kidney. Muscle and blood were assumed to represent, respectively, 43 and 8.5 % of the body weight [46]. Gabaculine (50 mg/kg) administration is indicated by the arrows.

Table 1. Effect of a mixture of insulin and glucose on the accumulation of ornithine in muscle induced by gabaculine

Two groups of 12 mice fed on Arg⁺ or Arg⁻ diets for 2 weeks were used. On the day of the experiment, 3 h after food removal, six mice from each group were injected intraperitoneally with 50 mg of gabaculine/kg in saline, and the remaining mice were injected with saline alone. After 30 min, 1 ml of 30 % (w/v) glucose containing 0.75 units of insulin was administered intraperitoneally to half of the mice treated either with gabaculine or with saline. All mice were killed 3.5 h later and muscular ornithine was assayed. *P* values denote statistical differences (*t* test) between mice treated and not treated with insulin and glucose: NS, not significant.

Hormonal treatment	Ornithine (nmol/g)			
	Arg ⁺ diet		Arg ⁻ diet	
	No gabaculine	Treatment with gabaculine	No gabaculine	Treatment with gabaculine
None	33.3 ± 6.5	252.8 ± 34.4	31.5 ± 4.8	267.3 ± 24.4
Insulin + glucose	26.1 ± 4.3	171.9 ± 23.1	15.2 ± 0.5	101.1 ± 10.6
<i>P</i>	NS	NS	< 0.01	< 0.001

and that gabaculine blocked this catabolism. Part of the ornithine accumulated in the Arg⁺-diet group appears to derive from dietary arginine, for in this group ornithine generally increased earlier and to a greater extent than in the group deprived of arginine (Fig. 1). Some might accumulate at the expense of the pool of non-protein arginine. In the Arg⁺-diet group this pool decreased in the organs studied (Fig. 2). However, in the mice deprived of arginine this pool was much smaller and did not decrease whether or not gabaculine was injected (Fig. 2), excluding the derivation of ornithine in this case from non-protein arginine.

Administration of insulin and glucose did not influence OAT activity or its inhibition by gabaculine (results not shown), but it was associated, whether or not the mice were pretreated with gabaculine, with a decrease in the content of ornithine in muscle (Table 1), particularly when arginine was absent from the diet. Since in normal man insulin administration decreases protein degradation in the whole body [34] and in muscle [35], our results support a major role of tissue proteolysis in supplying ornithine under the present conditions.

The increase in free ornithine+arginine, caused by gabaculine, in all the organs of Arg⁻ mice analysed

Fig. 3. Accumulation of ornithine+arginine in response to gabaculine in the organs and tissues analysed (liver, kidney, muscle, blood and brain)

Points plotted are the differences between the total amount of ornithine+arginine in the gabaculine-treated (time of administration shown by arrow) and in the control mice. The data refer only to the group deprived of arginine, and were calculated from those in Fig. 2.

Fig. 4. Influence of gabaculine on the release of $^{14}\text{CO}_2$ from L-[1- ^{14}C]ornithine

On the day of the experiment, groups of three mice fed on Arg⁺ (○, ●) or Arg⁻ (□, ■) diet for 4 weeks were injected intraperitoneally, 3 h after food removal, with 0.25 ml of saline alone (●, ■) or containing 50 mg of gabaculine/kg (○, □), followed 2 h later by 0.7 μCi (13 nmol) of L-[1- ^{14}C]ornithine in 0.1 ml of saline. $^{14}\text{CO}_2$ excretion was monitored for the indicated period as in [18]. No allowances have been made for recycling of metabolically generated $^{14}\text{CO}_2$, for in pilot experiments with $\text{NaH}^{14}\text{CO}_3$ > 95% of the radioactivity injected was excreted within 90 min as $^{14}\text{CO}_2$. At 6.5 h after administration of [^{14}C]ornithine the mice were decapitated, and the liver, kidney, brain, and samples of blood (collected from the neck) and muscle (from the hindlegs) were homogenized with 5 vol. of 1 M-HClO₄ as for the assay of ornithine and arginine (see the Experimental section). After centrifugation, the precipitate was washed with 1 M-HClO₄ and dissolved in 0.2 M-NaOH. Radioactivity was determined in supernatant and precipitate and is given in Table 2.

follows (Fig. 3), after a lag of about $\frac{1}{2}$ h (possibly the time for OAT inactivation), an approximately straight line, with a rate of ornithine+arginine accumulation of 1.15 $\mu\text{mol/h}$. This is a minimum estimate of the rate of ornithine catabolism in Arg⁻ mice if the aminotransferase were not inhibited, and represents, after extrapolation to the entire body weight and to a 24 h period, about 45 μmol of ornithine/day.

Respiratory excretion of $^{14}\text{CO}_2$ from [^{14}C]ornithine

The involvement of OAT in ornithine catabolism is confirmed in Fig. 4. Of the radioactivity injected as [1- ^{14}C]ornithine in arginine-fed or -deprived mice, 56 and 40%, respectively, was lost within 2 h as $^{14}\text{CO}_2$, and this loss was drastically decreased by administration of gabaculine. Jung & Seiler [18] made similar observations in mice fed on standard laboratory chow, and showed that ornithine decarboxylase does not participate significantly in the release of $^{14}\text{CO}_2$ observed. Therefore the small excretion of $^{14}\text{CO}_2$ (Fig. 4) after gabaculine administration is probably due to residual OAT activity.

Gabaculine administration markedly increased the radioactivity retained in tissues after administration of [1- ^{14}C]ornithine (Table 2). When gabaculine was not used, most of the radioactivity was precipitated by HClO₄ (Table 2), suggesting that it was in the proteins, and only 14% of the HClO₄-soluble radioactivity was associated with basic amino acids (results not shown), as expected

Fig. 5. Release of $^{14}\text{CO}_2$ after administration of L-[U- ^{14}C]ornithine

A group of three mice fed on standard laboratory chow (○) was injected subcutaneously in the thigh with [U- ^{14}C]ornithine (0.8 μCi ; 2.9 nmol) in the morning of the experiment, and the joint release of $^{14}\text{CO}_2$ from the group [18] was monitored for 5 h. A group of three mice fed on Arg⁻ diet was treated similarly, but the joint release of $^{14}\text{CO}_2$ from the group was only measured for the initial 2 h (●). The stippled area represents the range of $^{14}\text{CO}_2$ release expected from C-1 of [U- ^{14}C]ornithine, calculated from the results in Fig. 4 for mice not treated with gabaculine. Pilot experiments with mice fed on normal rodent chow showed no substantial difference in the kinetics of $^{14}\text{CO}_2$ release from labelled ornithine, whether it was administered subcutaneously or intraperitoneally.

Table 2. Influence of gabaculine administration and type of diet on the radioactivity in the tissues of mice 6.5 h after L-[1-¹⁴C]ornithine administration

For details see legend to Fig. 4. *P* values are for significant differences for each type of diet, between the groups treated and not treated with gabaculine.

Radioactivity in tissues	Radioactivity (d.p.m./g or d.p.m./ml)					
	Arg ⁺ diet			Arg ⁻ diet		
	No gabaculine	Treatment with gabaculine	<i>P</i>	No gabaculine	Treatment with gabaculine	<i>P</i>
Muscle						
Total	6442 ± 3213	27 624 ± 2954	< 0.0025	5545 ± 491	28 705 ± 5743	< 0.0025
HClO ₄ -soluble	1121 ± 276	23 098 ± 5052	< 0.0025	876 ± 168	23 465 ± 10 499	< 0.0125
Liver						
Total	38 358 ± 4550	100 778 ± 18 765	< 0.0025	55 704 ± 3434	87 115 ± 4928	< 0.0005
HClO ₄ -soluble	2034 ± 163	18 068 ± 913	< 0.0005	1849 ± 107	9124 ± 1394	< 0.0005
Kidney						
Total	25 183 ± 3763	50 537 ± 649	< 0.0005	33 003 ± 2224	53 298 ± 5369	< 0.0025
HClO ₄ -soluble	2172 ± 713	6352 ± 493	< 0.0025	2356 ± 256	4159 ± 501	< 0.005
Blood						
Total	12 025 ± 2108	24 843 ± 5950	< 0.0125	16 534 ± 2930	29 937 ± 2184	< 0.0025
HClO ₄ -soluble	587 ± 235	5108 ± 616	< 0.0005	717 ± 130	4891 ± 882	< 0.0025
Brain						
Total	3828 ± 435	13 654 ± 216	< 0.0005	4974 ± 162	9702 ± 1861	< 0.01
HClO ₄ -soluble	470 ± 128	3930 ± 909	< 0.0025	593 ± 188	2251 ± 834	< 0.025

if ornithine was metabolized to other amino acids. Gabaculine dramatically increased the HClO₄-soluble radioactivity, particularly in muscle (Table 2), and also the proportion of radioactivity in basic amino acids (90 %; results not shown), indicating that this inhibitor of OAT prevents the conversion *in vivo* of ornithine into glutamate, proline and secondary metabolites of these amino acids.

Nearly 30 % of the radioactivity injected as [U-¹⁴C]-ornithine was released within 2 h as ¹⁴CO₂ (Fig. 5), whether or not arginine was present in the diet. The rate of release of ¹⁴CO₂, followed in detail in the Arg⁺ group, declined rapidly. At 22 h from administration of the [U-¹⁴C]ornithine the mice were placed again in the CO₂-trapping system, and the radioactivity expired in 1 h was, in both groups, only about 0.25 % of that injected (results not shown). The release of ¹⁴CO₂ during the rapid phase exceeded by about 3-fold that expected if it were derived only from C-1 of ornithine, indicating extensive oxidation of the carbon skeleton of this amino acid. Indeed, irrespective of the type of diet, only about 20 % of the radioactivity remained in the animal after 6 days (results not shown; the ground carcass was extracted with 10 vol. of 1 M-NaOH and then with 5 vol. of 1 % SDS; radioactivity was also determined in the residue).

Labelling *in vivo* of arginine and ornithine from [1-¹⁴C]glutamate: influence of gabaculine

At 2 h after administration of [1-¹⁴C]glutamate to mice fed on the Arg⁻ diet, about 10 % of the radioactivity was recovered in the hydrolysate of the carcass (Table 3). About one-fifth of this radioactivity was not associated with amino acids (sample + wash, Table 3; see the last paragraph of the Experimental section); about three-

Table 3. Influence of gabaculine on the radioactivity in hydrolysates of the carcasses and in fractions thereof after administration of L-[1-¹⁴C]glutamate to mice deprived of arginine

Results are percentages of the radioactivity injected (5 µCi/mouse). Significant differences (*t* test; *n* = 4 per group) between untreated and gabaculine-treated mice are **P* < 0.025, ***P* < 0.01 and ****P* < 0.001.

Fraction	Radioactivity (%)	
	Without gabaculine	With gabaculine
Hydrolysate	9.73 ± 0.97	13.40 ± 0.35
AG 50W fractions		
Sample + wash	2.10 ± 0.50	2.48 ± 0.06
10 % Pyridine	7.00 ± 0.70	10.40 ± 0.34***
0.2 M-Pyridine acetate	0.12 ± 0.05	0.05 ± 0.01*
0.6 M-Pyridine acetate	0.21 ± 0.07	0.08 ± 0.02**

quarters co-eluted with glutamate, aspartate and neutral amino acids (10 %-pyridine fraction); and a small but substantial proportion (about 5 % of the radioactivity in the 10 %-pyridine fraction) was found in the two pyridine acetate fractions. As only basic amino acids are eluted in these two fractions, and lysine is not synthesized in mammals [36], radioactivity from glutamate was incorporated into ornithine and/or arginine. This was shown by rechromatography of the pyridine acetate fractions through AG 50W columns, and also by incubation with carbamoyl phosphate in the presence of arginase and ornithine transcarbamylase. The latter

treatment converted arginine into citrulline (shown by h.p.l.c.) and, correspondingly, the radioactivity was eluted on rechromatography in the 10%-pyridine fraction (results not shown).

Gabaculine markedly decreased the radioactivity in the pyridine acetate fractions (Table 3). This was not due to decreased recovery of ornithine or arginine (arginine, 2.9 ± 0.3 and 2.5 ± 0.1 mmol/mouse, and ornithine 0.060 ± 0.015 and 0.066 ± 0.035 mmol/mouse, in the control and gabaculine-treated groups respectively) or to decreased retention of labelled glutamate; in fact, the radioactivity in the 10%-pyridine fraction was increased by gabaculine (Table 3). Therefore the decrease in the radioactivity in ornithine and arginine induced by gabaculine appears to reflect a decrease in the synthesis *de novo* of these two amino acids, and thus strongly supports the role of OAT in their synthesis.

Amounts of intermediates and enzymes potentially involved in the synthesis of ornithine from acetylglutamate

Acetylglutamate was present in liver (70 nmol/g) and small intestine (27 nmol/g). In contrast with what would be expected if ornithine were derived from acetylglutamate, we failed to detect, in these tissues from mice deprived of arginine, acetylornithine (detection limit 2 nmol/g; $n = 3$), acetylglutamate kinase (assayed in freeze-thawed or Triton X-100-lysed mitochondria with and without desalting through Sephadex G-25; detection limit 0.1 nmol of product/min per g; $n = 3$) and acetylornithine aminotransferase (detection limit 30 nmol of product/min per g; $n = 6$). Therefore these results do not support the existence of the pathway from acetylglutamate to ornithine in the mouse.

DISCUSSION

We have confirmed [2,3] in the living mouse the synthesis of ornithine and arginine from [$1-^{14}\text{C}$]glutamate and demonstrated the involvement of OAT by showing that gabaculine, a potent and irreversible inhibitor of this enzyme [18,31], drastically decreased the radioactivity incorporated in these amino acids. The inhibition of OAT was more complete than the decrease in the labelling of ornithine and arginine, as expected if the reaction catalysed by OAT was not rate-limiting for the biosynthesis of ornithine. This agrees with the finding [37] of much less pyrroline-5-carboxylate synthetase than OAT activity in the intestine.

Our failure to detect in liver and intestine acetylornithine, acetylglutamate kinase and acetylornithine δ -aminotransferase is to be expected if a hypothetical route from acetylglutamate to ornithine [4-7] is not operative in mammals, and agrees with the involvement of OAT in ornithine synthesis. Acetylglutamate synthesis in mammals therefore appears to be a dead-end process for control of ureogenesis [14]. The fact that acetylglutamate synthetase is activated by arginine in mammals [38] would certainly be in conflict with a role in the synthesis *de novo* of ornithine and arginine.

The marked decrease in $^{14}\text{CO}_2$ release from [^{14}C]ornithine and the accumulation of ornithine after gabaculine treatment show that OAT is involved also in the degradation of ornithine. The accumulation of ornithine in mice deprived of arginine may appear to contradict the involvement of OAT in the biosynthesis of

ornithine. However, it is quite possible that proteolysis of tissue proteins could be the source of this ornithine; in the 9 h after administration of gabaculine the oxidation of 20 mg of tissue protein, a negligible amount by itself and also in terms of energy [about 0.32 kJ (0.08 kcal), compared with a daily energy intake of about 60 kJ (15 kcal)], would account for the observed accumulation of ornithine. Our finding that mixtures of insulin and glucose markedly decrease this accumulation supports this view.

Although OAT is widely distributed, only the intestine has been shown to synthesize citrulline from glutamate [39,40]. In addition, and in contrast with liver, kidney and other tissues, the intestine contains pyrroline-5-carboxylate synthetase [9,10] and is devoid of pyrroline-5-carboxylate dehydrogenase [41]. Morris [37] found that the intestinal, but not the hepatic or renal, OAT activity is correlated inversely with the nutritional requirement for arginine in the cat and the rat, this requirement being much higher in the cat. Here we extend this correlation to the mouse, which, with no apparent growth requirement for arginine [19], has about 3 times more intestinal OAT per kg body wt. than the rat, whereas renal or hepatic contents are similar or lower respectively [37]. Nutritional and hormonal factors affect this activity in the intestine and in other organs differently ([42]; E. Alonso & V. Rubio, results not shown), and even distinct tissue-specific isoenzymic forms of OAT have been postulated [43]. All this supports the specialization of OAT in the intestine for ornithine synthesis, and in other organs for ornithine catabolism.

Since in mice deprived of arginine and not treated with gabaculine the amounts of arginine and ornithine remain approximately constant (see the Results section), with no negative nitrogen balance (results not shown), the degradation of about 45 μmol of ornithine/day (see Results) has to be compensated by an equivalent daily synthesis of ornithine *de novo*. This value is remarkably close to the value of about 6.5 $\mu\text{mol/h}$ per 100 g body wt. [40] (or for a 30 g mouse, about 50 $\mu\text{mol/day}$) extrapolated from the results of Windmueller & Spaeth [39,40] for the release of citrulline into the blood from vascularly perfused preparations of rat intestine or from autoperfused segments of rat jejunum *in vivo*. It also agrees with the demonstration [12], using mitochondria from rat intestinal mucosa, of ornithine synthesis from glutamate of 1-4 nmol/min per mg of mitochondrial protein, extrapolating to about 40-160 $\mu\text{mol/day}$ for a 30 g mouse.

The synthesis from glutamate and the reconversion into glutamate of about 45 μmol of ornithine/day in arginine-deprived mice is in fact a futile cycle and may seem wasteful. Nevertheless, since only one ATP molecule is consumed per turn of the cycle [9], the energy consumption would represent only 4 J (< 1 cal) per day, a negligible amount when compared with daily energy expenditure of 63 kJ (15 kcal). In many respects this cycle resembles that proposed by Häussinger *et al.* [44] for hydrolysis and synthesis of glutamine by periportal and perivenous hepatocytes respectively. The cycle for ornithine is peculiar, however, in that the same enzyme (although perhaps in different isoenzymic forms [43]), OAT, is responsible for both the synthesis and the degradation of ornithine. That the synthesis *de novo* of ornithine is important, even with a normal dietary supply of arginine, is supported by the demonstration that

selective inhibition of intestinal ornithine trans-carbamoylase caused severe growth retardation in rats whether or not arginine was supplied in the diet [45].

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REFERENCES

1. Scull, C. W. & Rose, W. C. (1930) *J. Biol. Chem.* **89**, 109–123
2. Depocas, S. F. & Bouthillier, L. P. (1951) *Rev. Can. Biol.* **10**, 289
3. Sallach, H. J., Koeppe, R. E. & Rose, W. C. (1951) *J. Am. Chem. Soc.* **73**, 4500
4. Cunin, R., Glansdorff, N., Piérard, A. & Stalon, V. (1986) *Microbiol. Rev.* **50**, 314–352
5. Davis R. H. (1986) *Microbiol. Rev.* **50**, 280–313
6. Morris, C. J., Thompson, J. F. & Johnson, C. M. (1969) *Plant Physiol.* **44**, 1023–1026
7. McKay, G. & Shargool, P. D. (1977) *Plant Sci. Lett.* **9**, 189–193
8. Strecker, H. J. (1965) *J. Biol. Chem.* **240**, 1225–1230
9. Wakabayashi, Y. & Jones, M. E. (1983) *J. Biol. Chem.* **258**, 3865–3872
10. Wakabayashi, Y., Henslee, J. G. & Jones, M. E. (1983) *J. Biol. Chem.* **258**, 3873–3882
11. Ross, G., Dunn, D. & Jones, M. E. (1978) *Biochem. Biophys. Res. Commun.* **85**, 140–147
12. Henslee, J. G. & Jones, M. E. (1982) *Arch. Biochem. Biophys.* **219**, 186–197
13. Valle, D. & Simell, O. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Fredrikson, D. S., Goldstein, J. L. & Brown, M. S., eds.), pp. 382–401, McGraw-Hill, New York
14. Shigesada, K. & Tatibana, M. (1971) *J. Biol. Chem.* **246**, 5588–5595
15. Grisolia, S. & Cohen, P. P. (1953) *J. Biol. Chem.* **204**, 753–757
16. Van Dijk, M. & Lund, P. (1984) *Biochem. J.* **222**, 837–838
17. Lund, P. & Wiggins, D. (1986) *Biochem. J.* **239**, 773–776
18. Jung, M. J. & Seiler, N. (1978) *J. Biol. Chem.* **253**, 7431–7439
19. Bell, J. M. & John, A. (1981) *J. Nutr.* **111**, 525–530
20. Rogers, Q. R. & Harper, A. E. (1965) *J. Nutr.* **87**, 267–273
21. Shigesada, K., Aoyagi, K. & Tatibana, M. (1978) *Eur. J. Biochem.* **85**, 385–391
22. Alonso, E. & Rubio, V. (1983) *Eur. J. Biochem.* **135**, 331–337
23. Herzfeld, A. & Knox, W. E. (1968) *J. Biol. Chem.* **243**, 3327–3332
24. Vogel, H. J. & Jones, E. E. (1970) *Methods Enzymol.* **17A**, 260–264
25. Greenawalt, J. W. (1974) *Methods Enzymol.* **31**, 310–323
26. Wolf, E. C. & Weiss, R. L. (1980) *J. Biol. Chem.* **255**, 9189–9195
27. Nuzum, C. T. & Snodgrass, P. J. (1976) in *The Urea Cycle* (Grisolia, S., Báguena, R. & Mayor, F., eds.), pp. 325–349, John Wiley and Sons, New York
28. Alonso, E. & Rubio, V. (1985) *Anal. Biochem.* **146**, 252–259
29. Waters AUTO.TAG OPA Precolumn Derivatization Technique (1982) Waters Technical Bulletin, Waters Associates, Milford, MA
30. Volpe, P., Sawamura, R. & Strecker, H. J. (1969) *J. Biol. Chem.* **244**, 719–726
31. Yasuda, M., Toyama, S., Rando, R. R., Esaki, N., Tanizawa, K. & Soda, K. (1980) *Agric. Biol. Chem.* **44**, 3005–3006
32. Aniento, F., García-España, A., Portolés, M., Alonso, E. & Cabo, J. R. (1988) *Mol. Cell. Biochem.* **79**, 107–111
33. Rando, R. R. & Bangerter, F. W. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1276–1281
34. Fukagawa, N. K., Minaker, K. L., Rowe, J. W., Goodman, M. N., Matthews, D. E., Bier, D. M. & Young, V. R. (1985) *J. Clin. Invest.* **76**, 2306–2311
35. Gelfand, R. A. & Barret, E. J. (1987) *J. Clin. Invest.* **80**, 1–6
36. Bender, D. A. (1975) *Amino Acid Metabolism*, pp. 112–142, John Wiley and Sons, New York
37. Morris, J. G. (1985) *J. Nutr.* **115**, 524–531
38. Tatibana, M., Shigesada, K. & Mori, M. (1976) in *The Urea Cycle* (Grisolia, S., Báguena, R. & Mayor, F., eds.), pp. 95–105, John Wiley and Sons, New York
39. Windmueller, H. G. & Spaeth, A. E. (1975) *Arch. Biochem. Biophys.* **171**, 662–672
40. Windmueller, H. G. & Spaeth, A. E. (1981) *Am. J. Physiol.* **241**, E473–E480
41. Herzfeld, A., Mezl, V. A. & Knox, W. E. (1977) *Biochem. J.* **166**, 95–103
42. Sanada, Y., Suemori, I. & Katunuma, N. (1970) *Biochim. Biophys. Acta* **220**, 42–50
43. Volpe, P., Menna, T. & Pagano, G. (1974) *Eur. J. Biochem.* **44**, 455–458
44. Häussinger, D., Gerok, W. & Sies, H. (1984) *Trends Biochem. Sci.* **9**, 300–302
45. Hoogenraad, N., Totino, N., Elmer, H., Wraight, C., Alewood, P. & Johns, R. B. (1985) *Am. J. Physiol.* **249**, G792–G799
46. Humphrey G. F. (1971) in *Biochemists' Handbook* (Long, C., ed.), p. 639, E. and F. N. Spon, London

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