

Putrescine biosynthesis in mammalian tissues

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L-Ornithine decarboxylase provides *de novo* putrescine biosynthesis in mammals. Alternative pathways to generate putrescine that involve ADC (L-arginine decarboxylase) occur in non-mammalian organisms. It has been suggested that an ADC-mediated pathway may generate putrescine via agmatine in mammalian tissues. Published evidence for a mammalian ADC is based on (i) assays using mitochondrial extracts showing production of ¹⁴CO₂ from [1-¹⁴C]arginine and (ii) cloned cDNA sequences that have been claimed to represent ADC. We have reinvestigated this evidence and were unable to find any evidence supporting a mammalian ADC. Mitochondrial extracts prepared from freshly isolated rodent liver and kidney using a metrizamide/Percoll density gradient were assayed for ADC activity using L-[U-¹⁴C]-arginine in the presence or absence of arginine metabolic pathway inhibitors. Although ¹⁴CO₂ was produced in substantial amounts, no labelled agmatine or putrescine was detected. [¹⁴C]Agmatine

added to liver extracts was not degraded significantly indicating that any agmatine derived from a putative ADC activity was not lost due to further metabolism. Extensive searches of current genome databases using non-mammalian ADC sequences did not identify a viable candidate ADC gene. One of the putative mammalian ADC sequences appears to be derived from bacteria and the other lacks several residues that are essential for decarboxylase activity. These results indicate that ¹⁴CO₂ release from [1-¹⁴C]arginine is not adequate evidence for a mammalian ADC. Although agmatine is a known constituent of mammalian cells, it can be transported from the diet. Therefore L-ornithine decarboxylase remains the only established route for *de novo* putrescine biosynthesis in mammals.

Key words: agmatine, arginine, arginine decarboxylase, ornithine, polyamine.

INTRODUCTION

Polyamines are ubiquitous components of mammalian tissues that play essential roles in growth and signal transduction [1–4]. The polyamine biosynthetic pathway is established as a valid target for the synthesis of drugs useful for treatment of parasitic diseases, neoplasia and cancer chemoprevention. Putrescine is an essential precursor of the higher polyamines, spermidine and spermine. The pathway for mammalian putrescine synthesis was demonstrated to occur via the decarboxylation of ornithine, and ODC (L-ornithine decarboxylase) is a well-characterized and much studied enzyme [1,5]. Alternate pathways to putrescine biosynthesis from L-arginine are well known to occur in microbes [6,7], plants [8,9] and other organisms including animal and plant pathogens [10,11]. These occur via the direct decarboxylation of arginine to generate agmatine, which is then converted into putrescine either directly by agmatinase (agmatine ureohydrolase) in some micro-organisms such as *Escherichia coli* [6,12] or in a two-step process involving agmatine deiminase and *N*-carbamoyl-putrescine hydrolase in other micro-organisms such as *Pseudomonas aeruginosa* [7] and plants [9,13,14] (Figure 1). These pathways represent an important major route to polyamine biosynthesis in these organisms. Pyridoxal 5'-phosphate (PLP)-dependent ADC (L-arginine decarboxylase) enzymes, which were first described by Gale [15], have been fully characterized from many non-mammalian species including bacteria [16–18] and plants [19–21]. Recently, a pyruvoyl-dependent ADC from *Methanococcus jannaschii* was cloned, expressed and its structure determined [22,23].

It has been suggested that ADC may also occur in mammalian cells [24,25]. Several comprehensive review articles have described ADC as a component of the pathways metabolizing arginine [26–28]. There is evidence that agmatine may act as a

neurotransmitter [24,27–30] and the presence of ADC as a means of generating agmatine for this purpose is of obvious importance. However, since agmatine can also serve as a precursor of polyamines and agmatinase is established as an expressed mammalian gene product [31,32], the existence of a mammalian ADC also has important implications for polyamine metabolism. The regulation of polyamine synthesis is widely believed to be controlled by factors influencing ODC [1,4,33] and therapeutic interventions aimed at blocking polyamine synthesis frequently employ the use of inhibitors of ODC [34]. Significant putrescine production from arginine by ADC would provide an alternative route. Similarly, the presence of an ADC-regulated pathway to putrescine would also need to be considered in the interpretation of studies in which transgenic approaches to increase or decrease ODC have been used to derive mouse models to evaluate the role of polyamines in normal and neoplastic growth [35].

There is conclusive evidence for the presence of agmatine in mammalian tissues [24,28,36–38]. However, this may occur through uptake of the amine from dietary sources since agmatine is a known plant constituent and component of bacteria that may form part of the intestinal flora. Published evidence for the existence of a mammalian ADC reports enzyme activity associated with mitochondrial membranes and measurements of ¹⁴CO₂ release using [1-¹⁴C]arginine as the substrate [24,25,39]. Other cited evidence include a partial ADC clone sequence that was reported in rat kidney [40] and the recent identification of a protein as an ADC [41], which was previously identified as an inactive human paralogue of ODC and termed ODC-p [42]. We have investigated these claims and cannot substantiate the presence of ADC. The release of ¹⁴CO₂ from [U-¹⁴C]arginine by mouse and rat mitochondrial extracts was not accompanied by the formation of [¹⁴C]agmatine and the putative ADC sequences either are derived from microbial contamination or are inactive. Thus, at present,

Abbreviations used: ADC, L-arginine decarboxylase; L-NAME, N^ω-nitro-L-arginine methyl ester; ODC, L-ornithine decarboxylase; PLP, pyridoxal 5'-phosphate.

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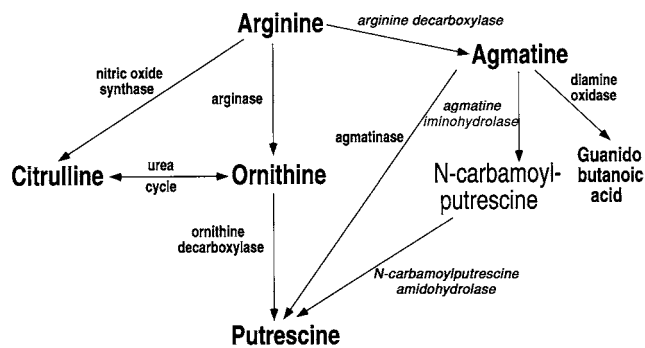


Figure 1 Arginine metabolic pathway

The enzymes and products shown in boldface and discussed in the text are known components of mammalian cells. Also shown in italics are ADC and the pathway to putrescine biosynthesis from L-arginine that occurs in plants and in *P. aeruginosa*. The indicated known mammalian enzymes are arginase (EC 3.5.3.1), ODC (EC 4.1.1.15), nitric-oxide synthase (EC 1.14.13.39), diamine oxidase (EC 1.4.3.6) and agmatinase (agmatine ureohydrolase, EC 3.5.3.11). The other enzymes shown in italics include ADC (EC 4.1.1.19), agmatine deiminase (agmatine iminohydrolase, EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (EC 3.5.1.53).

arginase activity followed by ODC remains the only validated route for the conversion of arginine into polyamines in mammalian tissues and mammalian agmatine may be derived solely from dietary sources.

MATERIALS AND METHODS

Materials

Metrizamide and Percoll used in density gradients to prepare mitochondrial extracts were purchased from Sigma (St. Louis, MO, U.S.A.). ADC from *E. coli* and arginine metabolic pathway inhibitors were from Sigma. L-[U-¹⁴C]Arginine (specific radioactivity from different lots ranged over 310–348 mCi/mmol) was purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). Protease inhibitor cocktail set 1 [500 μM 4-(2-aminoethyl)-benzenesulphonyl fluoride/HCl/150 nM aprotinin/1 μM E-64/0.5 mM EDTA/1 μM leupeptin at 1× concentration] was obtained from Calbiochem (San Diego, CA, U.S.A.). Liquid-scintillation fluids Econofluor-2 used for non-aqueous samples and Flo-Scint II used for the detection of aqueous radioactive products by HPLC were purchased from Packard Bioscience (Meriden, CT, U.S.A.). [U-¹⁴C]Toluene (4 × 10⁵ d.p.m./ml) and [7-¹⁴C]benzoic acid (5.4 × 10³ d.p.m./mg) radioactivity standards from New England Nuclear (Boston, MA, U.S.A.) were used to estimate the efficiency of counting ¹⁴C in the respective scintillation solutions.

Isolation of mitochondria from rodent tissues

Mitochondrial extracts were prepared from freshly isolated mouse (C57Bl/6) or rat (Sprague–Dawley) liver and kidney using a hybrid Percoll/metrizamide discontinuous density gradient as described previously [43]. Briefly, all solutions were prechilled and fed through an 18-gauge needle down the side of a 16 ml Beckman 3117-0160 centrifuge tube to form three layers from bottom to top: 2 ml of 35% (w/v) metrizamide in 0.25 M sucrose, 2 ml overlay of 17% (w/v) metrizamide in 0.25 M sucrose and 5 ml overlay of 6% (v/v) Percoll in 0.25 M sucrose. Tissues were homogenized in ice-cold Hepes/sucrose buffer (5 mM Hepes, pH 7.4/2 mM dithiothreitol/0.2 mM EDTA/0.25 M sucrose containing 1× protease inhibitors) using a Teflon-glass homogenizer. The homogenized extract was centrifuged at 1000 *g*_{av} for 10 min

to collect a post-nuclear supernatant that was centrifuged at 12000 *g*_{av} for 20 min to produce a crude mitochondrial pellet. The resulting pellet was resuspended in 4.8 ml Hepes/sucrose buffer and loaded on to the Percoll/metrizamide discontinuous density gradient described above. The gradient solution was centrifuged at 48000 *g*_{av} for 15 min to form discrete bands using slow acceleration–deceleration rates as recommended. The mitochondrial fraction that sedimented at the 17/35% metrizamide interface was collected and washed three times with 5 mM Hepes buffer (pH 7.4) and resuspended in 5 mM Hepes (pH 8.7) before assay for putative ADC activity. The protein content of the extract was determined by the Bradford method [44] using BSA (fraction V) as a standard.

Enzyme assay

Fractionated extracts were assayed for putative ADC activity by a modification of the assay described in [25]. This modified assay substituted [U-¹⁴C]arginine for [1-¹⁴C]arginine in the enzyme reaction mix. The reaction was performed in 250 μl containing 10 mM Tris/HCl (pH 8.2), 0.1 mM PLP, 1 mM dithiothreitol, protease inhibitor cocktail at 1× concentration, 0.2 mM EDTA, 1.0 mM MgSO₄ and 0.4 μCi of [U-¹⁴C]arginine (specific radioactivity, 310–348 mCi/mmol) for times up to 1 h at 30 °C. The reaction was performed in glass tubes with rubber stoppers that supported a central well containing hyamine hydroxide to collect ¹⁴CO₂ evolved during the incubation. The reaction was stopped by the addition of 50 μl of 40% (w/v) trichloroacetic acid injected through the rubber stopper using a syringe. The tubes were returned to the water bath for an additional 30 min to trap the CO₂ after which the central wells were transferred to scintillation vials and counted by liquid-scintillation counting using Econofluor[®] scintillation solution. Samples were spiked with a known amount of a toluene C-14 standard to determine the counting efficiency of ¹⁴C in Econofluor. A counting efficiency of 89% was used to convert c.p.m. into d.p.m. of ¹⁴CO₂ released.

HPLC analysis

The assay mix remaining in the glass tubes was collected and stored at –20 °C until examined by HPLC for the presence of [¹⁴C]agmatine. Trichloroacetic acid extracts described above were analysed by reverse-phase HPLC using a standard separation of polyamines [45] coupled with a Canberra Packard A140 radio-metric detector. The efficiency of counting [¹⁴C]agmatine formed in reactions using *E. coli* ADC was estimated to be 70% based on comparing the total radioactivity recovered as c.p.m. at the end of the HPLC run with a known number of d.p.m. injected into the HPLC column. This counting efficiency was used to convert peaks of [¹⁴C]agmatine integrated as c.p.m. into d.p.m. A reaction using *E. coli* ADC (0.025 unit) and 0.4 μCi of L-[U-¹⁴C]arginine was used as a standard to determine the retention time of [¹⁴C]agmatine on the HPLC column as illustrated in Figure 2.

Cloning of human ODC-like protein (ODC-p)

An open reading frame corresponding to human ODC-p [42] was isolated using the IMAGE clone 4156927 (Open Biosystems, Huntsville, AL, U.S.A.) as a template for PCR. Oligodeoxynucleotide primers (Macromolecular Core Facility, Hershey Medical Center) were designed to introduce *Sph*I (5'-CTCCTGCAAGGCGCATGCGGCTACCTGAGTG-3') and *Hind*III (5'-GGGGGAACGAGAAGCTTTCACATGATGCTC-3') restriction sites at the 5'- and 3'-ends of the cDNA respectively. The PCR product was digested and ligated into the same sites of the pQE-30 *E. coli* protein expression vector (Qiagen, Valencia,

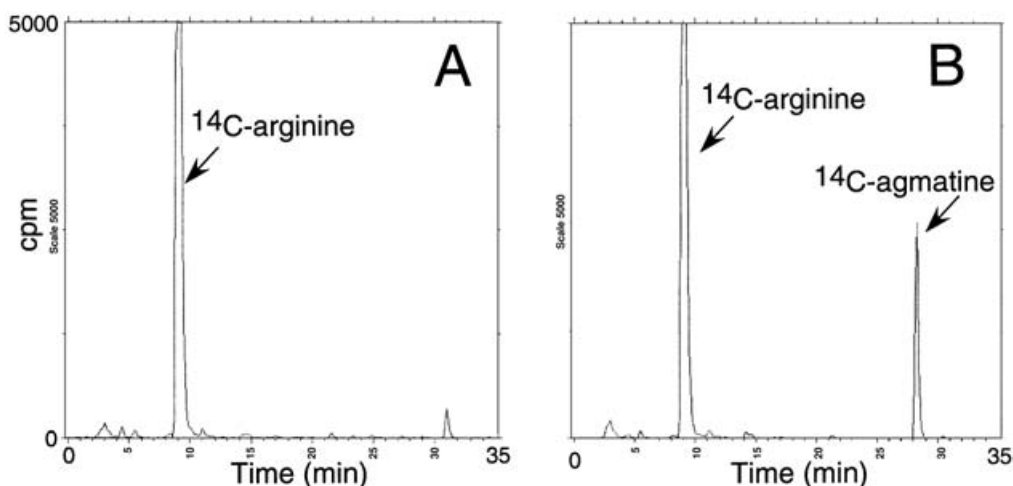


Figure 2 Production of [^{14}C]agmatine from [^{14}C]arginine by *E. coli* ADC

E. coli ADC (0.025 unit) was incubated with [^{14}C]arginine and assayed for ADC activity ($^{14}\text{CO}_2$ release and [^{14}C]agmatine formation) as described in the Materials and methods section. The trichloroacetic acid-soluble supernatants containing [^{14}C]arginine and [^{14}C]agmatine were resolved by HPLC with radiochemical detection. (A) Reaction containing extract and [^{14}C]arginine, which was stopped by the addition of trichloroacetic acid at zero time. The ratio of $^{14}\text{CO}_2$ released (15 540 d.p.m.) to total [^{14}C]agmatine formed (74 348 d.p.m.) after a 60 min incubation (B) was approx. 1: 5.

CA, U.S.A.) and transformed into Epicurian ColiTM XL1-Blue cells. The sequence of the derived clone was verified by DNA sequencing (Macromolecular Core Facility, Hershey, PA, U.S.A.). Recombinant ODC-p was expressed in transformed Epicurian ColiTM XL1-Blue cells grown at 37 °C in Luria-Bertani medium, and extracts were prepared 3 h after induction by 1 mM isopropyl β -D-thiogalactopyranoside. ODC-p was found to form insoluble inclusion bodies when expressed under these conditions, and therefore sonicated extracts of *E. coli* transformed with either empty vector (pQE) or pQE-ODC-p were used to assay whole extracts for any increase in ODC or ADC activity.

RESULTS

Measurement of ADC activity using *E. coli* ADC and [^{14}C]arginine

The enzymic decarboxylation of arginine results in the generation of both CO_2 and agmatine. To investigate the presence of ADC activity in mouse and rat tissues, we chose to use L-[^{14}C]arginine as a substrate for the enzyme reaction to monitor stoichiometric amounts of $^{14}\text{CO}_2$ and [^{14}C]agmatine produced as radioactive products derived from the same molecule. Decarboxylation involves the release of CO_2 from the C-1 position of [^{14}C]arginine generating [^{14}C]agmatine that will contain five labelled carbon atoms. *E. coli* ADC was used as a positive control for our studies, both to confirm the stoichiometry of the reaction and as a standard for detecting [^{14}C]agmatine in subsequent studies with mitochondrial extracts from mouse and rat tissues. $^{14}\text{CO}_2$ was assayed by trapping in alkali and the [^{14}C]agmatine determined using a radioactivity monitor to measure the products from HPLC separation. An example of a typical HPLC chromatogram showing the resolution of the reaction products produced after the incubation of *E. coli* ADC with [^{14}C]arginine is shown in Figure 2. [^{14}C]Arginine and [^{14}C]agmatine were well resolved with retention times of 8.5 and 28 min respectively. As shown in Table 1, the radioactivity associated with the agmatine peak when authentic ADC was used was approx. 4.7 times the measured $^{14}\text{CO}_2$ released, which is in close agreement (94%) with the expected 5-fold increase.

Measurement of $^{14}\text{CO}_2$ released after incubation of mouse liver mitochondrial extracts with [^{14}C]arginine

Preliminary experiments that used previously frozen mouse tissues resulted in only background levels of $^{14}\text{CO}_2$ release from [^{14}C]arginine. Therefore all data were collected after preparing mitochondrial extracts from freshly isolated tissues. Table 1 shows the results of two experiments in which freshly isolated mouse liver mitochondrial extract was used to monitor $^{14}\text{CO}_2$ release from [^{14}C]arginine. The release of $^{14}\text{CO}_2$ was substantial (as much as 664 000 d.p.m. in Experiment 1) but no formation of [^{14}C]agmatine was seen in any case where this was measured using HPLC analysis (Table 1 and Figure 3). The labelled metabolites derived from [^{14}C]arginine were eluted earlier in this analysis (Figure 3B). A conservative estimate of the limit of detection of the radioactivity monitor was approx. 420 d.p.m., which would correspond to a $^{14}\text{CO}_2$ release by ADC of 84 d.p.m. Furthermore, the release of $^{14}\text{CO}_2$ from the metabolism of [^{14}C]arginine by these extracts was considerably greater than the quantity released from the 1- ^{14}C atom alone. This indicates that additional oxidation of the arginine carbon backbone contributes to the observed $^{14}\text{CO}_2$ released by activities other than a putative ADC.

The possibility that [^{14}C]agmatine was formed but then degraded to other products was eliminated by the experiments shown in Figures 3(D), 3(E) and 4. Even when 1 mM unlabelled agmatine was added to trap any [^{14}C]agmatine formed by the mitochondrial extracts, no [^{14}C]agmatine could be observed (Table 1 and Figures 3D and 3E). Also, when [^{14}C]agmatine was added to the extracts, it was only slightly degraded (18% lost) in 1 h and this degradation was totally prevented by adding 1 mM unlabelled agmatine (Figure 4).

Similar studies were performed with mitochondrial extracts from mouse kidney and from rat liver and kidney. These are summarized in Table 2. Although, in all cases, there was a substantial release of $^{14}\text{CO}_2$, no [^{14}C]agmatine was detected. The possibility that ADC activity was present, but was not detected because of the rapid metabolism of the [^{14}C]arginine substrate by other enzymes, was examined by using inhibitors of these reactions. The conversion of [^{14}C]arginine into $^{14}\text{CO}_2$ was greatly

Table 1 Assay of mouse liver mitochondrial extract for putative ADC activity

Mouse liver mitochondrial extract was prepared using a metrizamide/Percoll density gradient and was assayed for putative ADC activity using [U-¹⁴C]arginine for the times indicated as described in the Materials and methods section. In experiment 1, a volume of 150 μ l of liver mitochondrial extract contained 580 μ g of total protein (3.9 μ g/ μ l). Each assay contained a total of 1 044 567 d.p.m. of [U-¹⁴C]arginine at the start of the incubation. In experiment 2, a volume of 100 μ l of liver mitochondrial extract contained 580 μ g of total protein (5.8 μ g/ μ l). Each assay contained a total of 1 011 152 d.p.m. of [U-¹⁴C]arginine at the start of the incubation. Identical extracts were treated in the absence or presence of 10 mM norvaline alone, with 10 mM norvaline + 1 mM agmatine or 10 mM norvaline + 1 mM agmatine + 1 mM aminoguanidine as indicated. In both experiments, *E. coli* ADC (0.05 unit/ μ l) was used as a positive control for detecting ADC activity as explained in the Materials and methods section. nd = not determined. Limit of detection = 420 d.p.m.

Extract	Volume assayed (μ l)	Time (min)	¹⁴ CO ₂ released (d.p.m.)	[¹⁴ C]Agmatine (d.p.m.)
Experiment 1				
<i>E. coli</i> ADC	0.5	0	0	0
	0.5	30	7480	nd
	0.5	60	14 478	67 369
Liver	150	0	14	< 420
	150	30	590 231	< 420
	150	60	664 524	nd
	50	60	280 074	nd
	100	60	597 674	nd
Experiment 2				
<i>E. coli</i> ADC	0.5	0	6	< 420
	0.5	60	15 540	74 348
Liver	100	0	0	< 420
	100	15	58 270	< 420
	100	30	85 655	nd
	100	60	160 846	< 420
	100	60	1600	< 420
Liver + norvaline	100	60	1321	< 420
Liver + norvaline + agmatine	100	60	1327	< 420
Liver + norvaline + agmatine + aminoguanidine	100	60	1327	< 420

inhibited by L-norvaline, an inhibitor of arginase (Table 1, experiment 2; Table 2 and Figure 3C) but was not affected significantly by L-NAME (N^ω-nitro-L-arginine methyl ester), an inhibitor of nitric oxide synthase, or aminoguanidine, an inhibitor of diamine oxidase (Table 2). This suggests that the ¹⁴CO₂ arises from oxidative metabolism of [¹⁴C]ornithine produced by arginase. The residual activity in the presence of L-norvaline, which amounts to less than 4% of the total, could be due to incomplete inhibition of arginase or to another pathway. In any event, the residual activity was also not due to ADC since no [¹⁴C]agmatine was detected (Tables 1 and 2 and Figure 3C).

Phylogenetic analysis of GenBank[®] ADC sequences

A mammalian ADC reported to be associated with mitochondria is commonly cited in the literature based on reports of the isolation by PCR, of a partial clone from rat kidney [40]. We used this reported sequence to generate a phylogenetic tree of encoded ADC protein sequences present in GenBank[®] databases. As shown in Figure 5, the encoded rat protein segregates with the sequences from *Azotobacter vinelandii* and *Pseudomonas sp.* and no sequence similar to that of the putative rat sequence was found in the available mammalian GenBank[®] databases. This suggests that the putative rat sequence is possibly the result of the amplification of a bacterial contaminant of the originally isolated rat kidney preparation.

Analysis of ODC-p

A human cDNA for a protein related to ODC was identified in 2001 and termed ODC-p [42]. ODC-p did not decarboxylate ornithine when expressed *in vitro* [42]. This protein has very recently been designated as an ADC [41]. We expressed recombinant ODC-p in *E. coli* and were unable to detect any ADC or ODC activity in the bacterial extracts (results not shown). This is not surprising since the sequence of ODC-p lacks several key residues that are essential for decarboxylase activity in ODC.

These include: (i) the residue equivalent to Cys-360 in ODC which is the essential proton donor for product release [46,47]; (ii) the residue equivalent to Asp-88 in ODC, which interacts with Lys-69 when the substrate binds releasing the PLP cofactor from Lys-69 [47,48]; (iii) Phe-400, which is part of the sequence forming the essential dimer interface [49,50]. Mutation of any one of these residues in ODC reduces ODC activity by several orders of magnitude. It is therefore most unlikely that any significant decarboxylase activity is associated with ODC-p.

DISCUSSION

Our results do not support the existence of a mammalian ADC. Although it is impossible to prove that such an enzyme does not exist, it is clear that the available evidence is insufficient to support claims of ADC. It has been known for many years that mitochondrial oxidation of L-[1-¹⁴C]ornithine can generate ¹⁴CO₂, and assays for ODC that use the production of ¹⁴CO₂ must use cytosolic extracts that lack contamination with ornithine transaminase to produce accurate results [51–53]. The data shown in Tables 1 and 2 indicate that ¹⁴CO₂ production from L-[U-¹⁴C]-arginine can also occur via the oxidation of L-[¹⁴C]ornithine generated by arginase activity. There are two forms of arginase: arginase I, which was supposed to be limited to liver and a few other tissues [26] but has recently been reported in many mouse tissues [54], and arginase II, which is a widespread mitochondrial enzyme [26,55]. Arginase activity can therefore lead to the production of ornithine and oxidation of this amino acid can liberate ¹⁴CO₂ from all of its C atoms. Any claim of ADC activity must be supported by unequivocal demonstration of the agmatine product.

Two previous reports of mitochondrial ADC in rat tissues did claim to identify agmatine as the product of the reaction [25,39]. We are unable to confirm these results and cannot explain the discrepancy. However, in one report [25], the identification of agmatine was made on the basis of the conversion of [³H]arginine (isotope-labelling position unspecified) into a product that

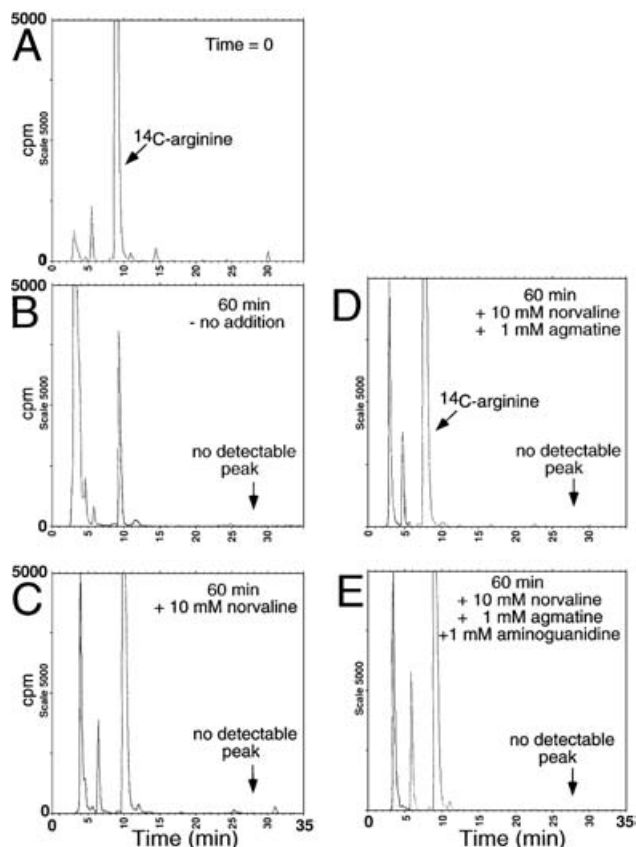


Figure 3 Lack of [^{14}C]agmatine production from [^{14}C]arginine by mitochondrial extracts

Trichloroacetic acid-soluble extracts from the mouse liver mitochondrial results shown in Table 2 were analysed by HPLC with radiochemical detection as described in the Materials and methods section. No peak corresponding to [^{14}C]agmatine was detected under any of the conditions analysed. Results are shown for samples incubated for 0 min (A), 60 min (B), 60 min with the addition of 10 mM norvaline (C), 60 min with the addition of 10 mM norvaline and 1 mM agmatine (D), and 60 min with the addition of 10 mM norvaline, 1 mM agmatine and 1 mM aminoguanidine (E).

co-migrated with agmatine (identified by staining with ninhydrin) on TLC [56]. The HPLC technique used in our studies is likely to be more precise than this TLC [56], and it is possible that other products obtained from oxidative metabolism of [^3H]arginine

Table 2 Assay of rat and mouse liver and kidney mitochondrial extracts for ADC activity

Mitochondrial extracts were prepared from freshly isolated rat and mouse liver or kidney and assayed for putative ADC activity as described in the legend to Table 1. Parallel assays were treated in the absence (none) or presence of 10 mM norvaline alone, 1 mM L-NAME alone, 1 mM aminoguanidine alone or in the presence of both 10 mM norvaline + 1 mM L-NAME as indicated. In this experiment, each assay contained 200 μg of total protein. Each assay contained a total of 1 043 067 d.p.m. (mouse) or 1 035 763 d.p.m. (rat) of [^{14}C]arginine at the start of the incubation. *E. coli* ADC was used as a positive control for detecting ADC activity as explained in the Materials and methods section. Limit of detection = 420 d.p.m.

Tissue	Addition	$^{14}\text{CO}_2$ released (d.p.m.)	[^{14}C]Agmatine (d.p.m.)
<i>E. coli</i> ADC	None	13 692	75 363
Rat liver	None	> 84 892*	< 420
Rat liver	Norvaline	2617	< 420
Rat liver	L-NAME	190 927	< 420
Rat liver	Aminoguanidine	202 431	< 420
Rat liver	Norvaline + L-NAME	2735	< 420
Rat kidney	None	22 072	< 420
Rat kidney	Norvaline	2278	< 420
Rat kidney	L-NAME	19 960	< 420
Rat kidney	Aminoguanidine	18 441	< 420
Rat kidney	Norvaline + L-NAME	4098	< 420
Mouse liver	None	60 943	< 420
Mouse liver	Norvaline	1255	< 420
Mouse liver	L-NAME	56 880	< 420
Mouse liver	Aminoguanidine	67 952	< 420
Mouse liver	Norvaline + L-NAME	1303	< 420
Mouse kidney	None	64 441	< 420
Mouse kidney	Norvaline	2319	< 420
Mouse kidney	L-NAME	30 535	< 420
Mouse kidney	Aminoguanidine	64 865	< 420
Mouse kidney	Norvaline + L-NAME	3248	< 420

* Value underestimated due to spillage of hyamine from the central well during transfer to a scintillation vial.

were not resolved from [^3H]agmatine in the earlier studies [25]. In the other report [39], HPLC separation of products from [^3H]arginine (isotope-labelling position and purity unspecified) after pre-column derivatization with *o*-phthalaldehyde was used, but the only metabolites found after a 75 min incubation with a crude rat kidney homogenate were agmatine (18.9%) and ornithine (34.3%), with 40.4% of the [^3H]arginine remaining. The actual chromatogram of labelled products was not shown; nor was the reproducibility of the observation indicated. This result is in striking contrast to our studies with [^{14}C]arginine, where

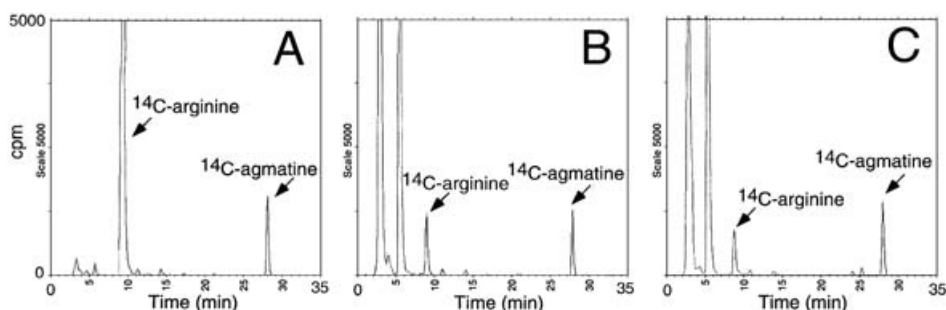


Figure 4 [^{14}C]Agmatine is not degraded extensively by mouse liver mitochondrial extracts

[^{14}C]Agmatine was prepared from a 60 min reaction containing 0.025 unit of *E. coli* ADC that was boiled at the end of the incubation to inactivate any residual *E. coli* ADC activity. An aliquot of this reaction was used to spike the following reactions containing 392 μg of liver mitochondrial extract that were then incubated at 30 $^\circ\text{C}$ for 60 min and stopped by the addition of trichloroacetic acid as described in the Materials and methods section. (A) Chromatogram from an incubation that contained mitochondrial extract that was boiled for 5 min before adding ADC assay mix + [^{14}C]agmatine spike (7151 d.p.m.). (B) Same reaction as in (A) but using freshly prepared extract (not boiled) + [^{14}C]agmatine (5963 d.p.m.) and (C) same as in (B) with the addition of 1 mM unlabelled agmatine + [^{14}C]agmatine (7049 d.p.m.).

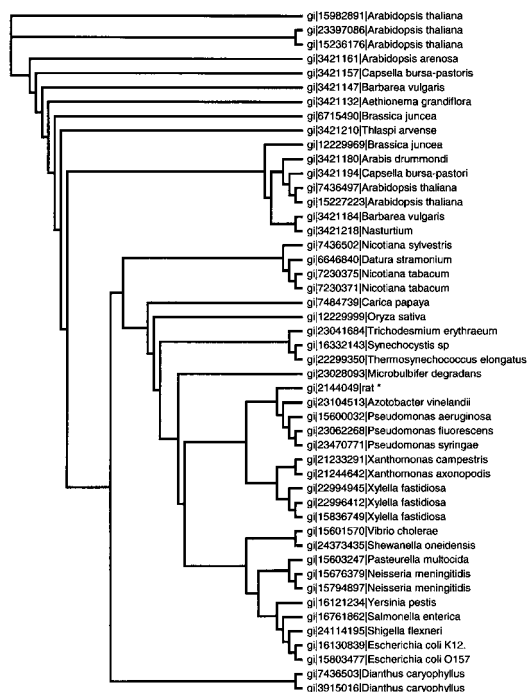


Figure 5 Phylogenetic tree of aligned ADC sequences

The phylogenetic tree was calculated from aligned ADC sequences using the program Clustal W and was displayed with the program Treeview [68]. *, Putative rat ADC.

the majority of the arginine was converted into multiple products that elute early from our HPLC system and do not correspond to either agmatine or ornithine (see Figure 3).

Our results are not in agreement with a recent report that 15–20% of arginine uptake in isolated perfused rats' livers was converted into agmatine [57]. However, that paper also reported that agmatine levels in the perfused livers were 29 nmol/g wet wt and were increased to 129 nmol/g wet wt after perfusion with 0.5 mM arginine. These values are 58 and 87% of the arginine concentration measured and three orders of magnitude higher than other reports of liver agmatine (e.g. liver agmatine in the rat was reported as 5.6 ng/g wet wt [38]) suggesting that the analysis is in error and/or that bacterial contamination leads to arginine metabolism.

Putative sequences encoding mammalian ADC are also not convincing. The 'partial clone' of ADC reported as isolated from rat kidney [40] has no obvious counterpart in the current mammalian genome databases and may result from bacterial contamination. Even though mammalian and trypanosomal ODC enzymes do have a very modest ability to act on arginine their active sites are strongly selective for ornithine [58,59]. It is not clear whether the modifications in amino acids present in the ODC-p sequence [42], recently described as ADC on the basis of its expression in COS-7 cells [41], would increase the likelihood of arginine being recognized. Structural and biochemical studies have identified key components needed for decarboxylase activity [47–50] and at least three of these are missing from the ODC-p protein. This is consistent with the absence of ODC or ADC activity in *E. coli* extracts that express the recombinant ODC-p. Thus it is most unlikely that physiological quantities of ODC-p would contribute to ADC activity. A more probable function for ODC-p is that it would act as an antizyme inhibitor protein, as first suggested by Pitkänen et al. [42]. Such activity may increase not only endogenous ODC activity but also the uptake of exogenous amines since antizyme not only reduces ODC content

but also down-regulates polyamine transport [33,60,61]. Since agmatine has been shown to be taken up by this transport system [62,63], the expression of ODC-p could increase agmatine levels in mammalian cells without having any ADC activity.

The evidence that agmatine is present in mammalian tissues is quite convincing [24,28,36–38] and human agmatinase has been cloned recently [31,32]. However, rather than being part of a polyamine biosynthetic pathway forming polyamines from arginine, this enzyme may serve to degrade agmatine from dietary sources. This would limit the levels of this amine and could serve a protective effect since agmatine may act as a neurotransmitter. Several studies have shown that agmatine can regulate polyamine synthesis via its ability to induce antizyme and spermidine/spermine *N*¹-acetyltransferase [64–67]. However, these studies performed in cultured cells use mM levels of agmatine and there is no evidence that these are physiologically relevant whether from ADC or dietary sources. At present, there is no convincing evidence that ADC or agmatine influences mammalian polyamine metabolism.

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