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METABOLISM OF AGMATINE IN FRUIT-BODIES OF THE FUNGUS *PANUS TIGRINUS* (BULL. EX FR.) SING.*

A. BOLDT, J. MIERSCH and H. REINBOTHE

University of Halle, Sektion Biowissenschaften, Fachbereich Biochemie, Biologische Abteilung, Halle (Saale), G.D.R.

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Abstract—The biosynthesis and degradation of agmatine (1-amino-4-guanidobutane) and arcain (1,4-diguanidobutane) in fruit-bodies of the fungus, *Panus tigrinus* (Bull. ex Fr.) Sing. (Tricholomataceae), have been investigated. Agmatine is produced either from L-arginine by decarboxylation, or from putrescine by transamidination. Activity of the L-arginine carboxy-lyase in the fruit-bodies is optimal at pH 5·2–5·4. Agmatine may be further amidinated to yield arcain. The hydrolytic cleavage of arcain by a ureohydrolase gives rise to agmatine, and agmatine is further hydrolysed to urea and putrescine. Three pathways of arginine catabolism have thus been demonstrated. The most important leads to γ-aminobutyric acid, with the intermediate formation of agmatine, putrescine and γ-aminobutyraldehyde (Δ^1-pyrroline).

INTRODUCTION

ALTHOUGH agmatine (1-amino-4-guanidobutane) appears to be widespread in nature, relatively little is known of the metabolism of this compound.[1–3] L-Arginine, which is the key metabolite in the formation of guanidines, gives rise to agmatine in a number of micro-organisms and also in higher plants.[4–6] Agmatine is an intermediary compound in putrescine synthesis from L-arginine in *Hordeum vulgare*, in which putrescine formation from agmatine is the result of two enzymatic steps: agmatine → carbamoylputrescine → putrescine.[7] In *Escherichia coli*, however, an agmatine-ureohydrolase is present, which cleaves agmatine to putrescine and urea.[8] Whether agmatine is involved in the formation of coumaroyl-agmatine, hordatine and the isoprenoid guanidines, is still a matter for speculation.[9,10]

Some time ago, we published the results of an investigation into the metabolism of γ-guanidobutyric acid in fruit-bodies of *Panus tigrinus*.[11] In that paper the presence of two other guanidine compounds besides arginine and γ-guanidobutyrate was briefly mentioned, which were tentatively identified as agmatine and arcain (1,4-diguanidobutane). More recently, one of us (J.M.) has reported the isolation and identification of arcain from fruit-bodies of *P. tigrinus*,[12] and, as far as we can ascertain, this is the first report of the occurrence

* This paper is dedicated to Professor Dr. K. Mothes at the occasion of his 70th birthday.

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[2] H. G. BOIT, *Ergebns. der Alkaloid-Chemie bis* 1960, Akademie Verlag, Berlin (1961).
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[4] J. MIERSCH, *Biol. Rdsch.* **6**, 72 (1968).
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[6] L. V. EGGLESTON, *Biochem. J.* **65**, 735 (1957).
[7] T. A. SMITH, *Phytochem.* **4**, 599 (1965).
[8] D. R. MORRIS and A. B. PARDEE, *J. Biol. Chem.* **241**, 3129 (1966).
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of arcain in plant tissue. Arcain was first isolated from the mussel, *Arca noae*,[13] and since then this compound has been found in other invertebrates.[14,15] There has been some speculation about arcain biosynthesis, although in animals arcain formation is known to be mediated by arginine-agmatine transamidination.[16]

The biosynthesis and degradation of agmatine and arcain in fruit-bodies of *P. tigrinus* (Bull. ex Fr.) Sing. is the subject of the present communication. A report of the course of arginine metabolism in *P. tigrinus* will be published elsewhere.[17]

RESULTS AND DISCUSSION

Feeding experiments. Fruit-bodies of *P. tigrinus* transform L-arginine-^{14}C to labelled γ-guanidobutyric acid,[11,18] agmatine, and arcain. The isolation of the latter two compounds, which are present in the fungal material in relatively small amounts, was only possible by adding appropriate quantities of the inactive carrier compounds. In order to investigate the course of arginine breakdown, the following labelled compounds were fed through the stipe of freshly harvested fruit-bodies for 2 hr: L-arginine-amidine-^{14}C, L-arginine-U-^{14}C, agmatine-U-^{14}C, and putrescine-1,4-^{14}C. As is seen from Table 1 feeding L-arginine-amidine-^{14}C results in the formation of radioactive γ-guanidobutyric acid and agmatine

TABLE 1. METABOLISM OF COMPOUNDS RELATED TO γ-AMINOBUTYRATE IN FRUIT-BODIES OF *Panus tigrinus* (BULL. EX. FR.) SING.*

Labelled compound fed	Substrate conc. (μmoles)	^{14}C added (μc)	Total radio-activity e/counts/min $\times 10^{-4}$	% Radioactivity in 70% ETOH extract†										
				Arg	Agm	Put	Orn	AB	GB	Urea	Asp	Glu	Glu-NH_2	Unk
L-Arg-amidine-^{14}C	3·0	4·0	2·27	54	10	—	—	—	6	4	—	—	—	23
L-Arg-U-^{14}C	3·0	4·0	3·09	24	9	3	2	12	9	2	2	3	3	31
Agmatine-U-^{14}C	9·0	1·0	1·18	—	57	2	—	3	9	—	—	3	—	25
Putrescine-1,4-^{14}C	3·0	4·0	1·75	—	—	40	—	22	—	—	4	4	1	28

* Compounds were fed through the stipe for 2 hr.

† Arg, arginine; Agm, agmatine; Put, putrescine; Orn, ornithine; AB and GB, γ-amino- and γ-guanidobutyric acid; Urea; Asp, aspartic acid; Glu, glutamic acid; Glu-NH_2, glutamine; Unk, unknown compounds.

as well as non-labelled ornithine. As observed previously,[11] the guanidobutyrate formed is partially degraded to γ-aminobutyrate (unlabelled) and urea. Urea-^{14}C is detectable only in trace amounts, probably because an active urease is present.[19] L-Arginine-U-^{14}C feeding results in the formation of both labelled γ-guanidobutyric acid and ornithine, indicating a transamidination reaction.[11] L-Amino-acid-oxidase is absent;[19] the presence of L-arginine-transamination reaction is speculative. Labelled γ-aminobutyric acid is found, suggesting its formation either from (a) ornithine or (b) via a pathway involving agmatine and putrescine

[13] F. KUTSCHER, D. ACKERMANN and O. FLÖSSNER, *Physiol. Chem.* **199,** 273 (1931).
[14] Y. ROBIN and J. ROCHE, *Comp. Biochem. Physiol.* **14,** 453 (1965).
[15] F. KUTSCHER and D. ACKERMANN, *Ann. Rev. Biochem.* **2,** 365 (1933).
[16] C. AUDIT, B. VIALA and Y. ROBIN, *Comp. Biochem. Physiol.* **22,** 775 (1967).
[17] H. REINBOTHE, J. MIERSCH and A. BOLDT, *4th Intern. Alcaloid Symposium*, Sonderband, Halle (1969).
[18] J. MIERSCH and H. REINBOTHE, *Flora* **156,** 543 (1966).
[19] J. MIERSCH, Diplomarbeit an der Martin-Luther-Universität, zu Halle-Wittenberg (1964).

as intermediary compounds. In fact L-arginine-U-^{14}C gave rise to labelled agmatine and putrescine (Table 1). To prove this hypothesis, agmatine-U-^{14}C and putrescine-1,4-^{14}C were fed to fruit-bodies. As is seen (Table 1), uniformly labelled agmatine is converted to radioactive putrescine, γ-aminobutyrate, and γ-guanidobutyrate. Putrescine-1,4-^{14}C is mainly transformed to γ-aminobutyric acid. In *P. tigrinus* therefore, L-arginine might be catabolized to γ-aminobutyrate via agmatine and putrescine as has been reported in bacteria.[5,8,20,21]

Experiments with Enzyme Preparations

To confirm the postulated reaction sequences, the metabolism of arginine, agmatine, and putrescine has been studied in crude enzyme preparations obtained from fruit-bodies of *P. tigrinus*.

(A) *Arginine Carboxy-layse*

An enzyme is present decarboxylating L-arginine to agmatine. Such an arginine carboxy-lyase (E.C. 4.1.1.19) is known from bacteria (*E. coli*,[22] *Aeromonas shigelloides*)[20] and from crude enzyme preparations from barley seedlings.[23] The crude dialysed *Panus* enzyme catalysed the formation of 20 μl CO_2/5 min/mg protein at pH 5·2 in 0·2 M Na-acetate buffer and at a substrate level of $1{\cdot}33 \times 10^{-3}$ M L-arginine. The dialysed enzyme preparation used was not capable of decarboxylating the following compounds: D-arginine, L-homoarginine, L-arginic acid, γ-guanidobutyric acid, L-glutamic acid, and L-ornithine. L-Canavanine was attacked at a rate of only 32% in comparison to L-arginine. The pH-optimum of arginine decarboxylation in our preparation is pH 5·2–5·4. Optimum temperature for the enzyme assay is 37–40°. These results agree with one of the respective optima reported for *E. coli*.[8] Added pyridoxal phosphate, which is the coenzyme for amino acid decarboxylases,[24] was without any effect in promoting carbon dioxide evolution from L-arginine.

There is no evidence that putrescine is formed in *Panus* from L-ornithine by direct decarboxylation. Further purification of L-arginine carboxy-lyase is in progress.

(B) *Agmatine Hydrolysis*

Degradation of the resulting agmatine is mediated by an agmatine ureohydrolase.[25] Such an enzyme is known from some micro-organisms.[20,26] From our recent investigation, there is most evidence that several different ureohydrolases exist in *Panus* fruit-bodies. One of them attacks arcain.[25]

(C) *Putrescine Oxidation*

In dialysed enzyme preparations from *P. tigrinus* fruit-bodies, a putrescine oxidizing enzyme has been demonstrated. As compared to the unsupplemented reaction mixture, the addition of 0·4 μmoles FAD increased putrescine oxidation about 33%, but by comparison, addition of 5 μmoles pyridoxal phosphate caused only a stimulation of 7%. The *Panus* enzyme catalyses both oxygen consumption (followed manometrically) and ammonia

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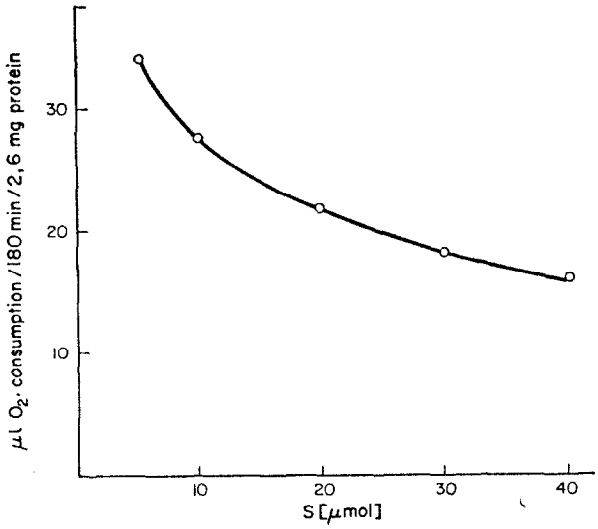

FIG. 1. PUTRESCINE OXIDIZING ENZYME ACTIVITY OF *Panus tigrinus* PLOTTED AGAINST SUBSTRATE CONCENTRATION. THE STANDARD INCUBATION MIXTURE CONTAINED: PUTRESCINE–DIHYDROCHLORIDE 5·0; 10·0; AND 40·0 µmoles, RESP.

liberation (estimated spectrophotometrically in a coupled optical test in which the liberated ammonia is consumed in glutamate formation by glutamic dehydrogenase).[27] Diamine oxidase converts putrescine to γ-aminobutyraldehyde, which is in equilibrium with Δ^1-pyrroline.[28] The Δ^1-pyrroline was trapped with *o*-aminobenzaldehyde as the quinazolidine derivative and then spectrophotometrically assayed. Carbonyl reagents added in a concentration of 5×10^{-7} M inhibited only slightly the reaction: hydroxylamine for 25%, 8-hydroxyquinoline for 13%, semicarbazide for 4%. There is a marked delay in enzyme activity when substrate or inhibitor concentrations are increased (Fig. 1), in contrast to the results reported for the putrescine oxidase of *Micrococcus rubens*.[29] The *Panus* enzyme catalysing putrescine breakdown is at present rather ill-defined and further work is needed to purify and characterize this enzyme.

(D) *γ-Aminobutyraldehyde Oxidation*

The conversion of γ-aminobutyraldehyde to γ-aminobutyric acid is mediated by a dehydrogenase which has been assayed spectrophotometrically at 366 nm by adding NAD to a crude enzyme preparation of *Panus*. The reaction was linear with time. Such an enzyme has been found in *Pseudomonas fluorescens*[30,31] and has been classified as γ-aminobutyraldehyde dehydrogenase (aldehyde–NAD–oxidoreductase, E.C.1.2.1.3). It is interesting that in pea seedlings and in baker's yeast, γ-aminobutyrate is not formed from arginine via agmatine, putrescine and γ-aminobutyraldehyde, but in a more direct way viz. from L-

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ornithine by δ-transamination and subsequent decarboxylation of the resulting keto analogue.[32]

(E) *Transamidination*

Our results show that agmatine, which is formed by decarboxylation of L-arginine or by hydrolysis of arcain is the precursor of putrescine and γ-aminobutyric acid in *Panus*. Agmatine synthesis is, however, also mediated by a group transfer to putrescine from a suitable amidine donor compound (Table 2), and arcain is formed from agmatine by transamidination. In an earlier publication concerning γ-guanidobutyric acid metabolism in

TABLE 2. FORMATION OF AGMATINE AND ARCAIN IN A CRUDE ENZYME PREPARATION OBTAINED FROM FRUIT BODIES OF *Panus tigrinus* (BULL. EX FR.) SING.

Compounds added	Substrate conc. (μmoles)	Specific radioactivity (mc/mmole)	Radioactive products	% Radioactivity in 70% in ETOH extract	Specific incorporation rate (%)
L-Arginine-amidine-^{14}C	5·0	1·00	Arginine	81	
+ agmatine	7·5	—	Agmatine	10	12
			Arcain	2	169
DL-Canavanine-amidine-^{14}C	5·2	0·96	Canavanine	89	
+ agmatine			Agmatine	2	4
	7·5	—	Arcain	5	Not determined
L-Arginine	5·0	—	Putrescine	99	
+ putrescine-1,4-^{14}C	7·2	0·69	Agmatine	tr.	Not determined
			Arcain	tr.	Not determined
DL-Canavanine-amidine-^{14}C	5·2	0·96	Canavanine	72	
+ putrescine	7·5	—	Agmatine	3	Not determined
			Arcain	4	182
γ-Guanidobutyric acid	5·0	—	Putrescine	98	
+ putrescine-1,4-^{14}C	7·2	0·69	Agmatine	1·5	13
			Arcain	tr	4

The incubation mixture contained: protein, 0·47 mg; amidine donor compound, 5·0–5·2 μmoles; amidine acceptor compound, 7·2–7·5 μmoles; mercaptoethanol, 1·0 μmole; phosphate buffer, 7·4, 20 μmoles; in a total volume of 0·8 ml. Incubation time: 1 hr at 32°.

tr. = traces (< 1% of total radioactivity).

Panus fruit-bodies, we reported the preparation of a crude enzyme exhibiting transamidinase activity (E.C.2.6.2).[11] L-Arginine, L-canavanine, γ-guanidobutyrate, and hydroxyguanidine were found to be active donor compounds of the amidine group, L-ornithine, L-canaline, γ-aminobutyrate, and agmatine functioned as suitable acceptor compounds in amidine

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group transfer. Dialysed enzyme preparations containing transamidinase, ureohydrolase, arginine carboxy-lyase and urease, were incubated with L-arginine-amidine-^{14}C (5 μmoles) and agmatine (7·5 μmoles). Both agmatine and arcain were found to be radioactive (Table 2). Agmatine labelling might be the result of two different reactions: decarboxylation of L-arginine and cleavage of arcain to agmatine and urea. Agmatine formation by arginine decarboxylation was eliminated using DL-canavanine-amidine-^{14}C instead of L-arginine-amidine-^{14}C (Table 2). In this case the introduction of radiocarbon in arcain was higher than in agmatine. Evidently, arcain in *Panus* is formed by transamidination. This is in accordance with the reaction shown in Equation 1.

Canavanine-amidine-^{14}C + agmatine → arcain-^{14}C + canaline

EQUATION 1.

The enzyme preparation catalysed agmatine and arcain formation from putrescine, with L-arginine, DL-canavanine, and γ-guanidobutyrate as amidine donor compounds (Table 2), suggesting a stepwise amidination of putrescine via agmatine yielding arcain. Such a mechanism is known from hirudonine biosynthesis in the invertebrate, *Hirudo medicinalis*[33] and from streptomycin formation in *Streptomyces griseus*.[34] The formation of agmatine and arcain from putrescine and L-arginine are quantitatively unimportant in *Panus* fruit-bodies when compared with the degradation of putrescine to γ-aminobutyrate as revealed from tracer experiments *in vivo* and *in vitro*. However, the relative importance of the multiple pathways in arginine degradation which may be termed 'γ-guanidobutyric acid pathway', 'putrescine pathway', and 'arcain pathway', have still to be established.

The probable course of agmatine metabolism in fruit-bodies of *P. tigrinus* is schematically presented in Fig. 2.

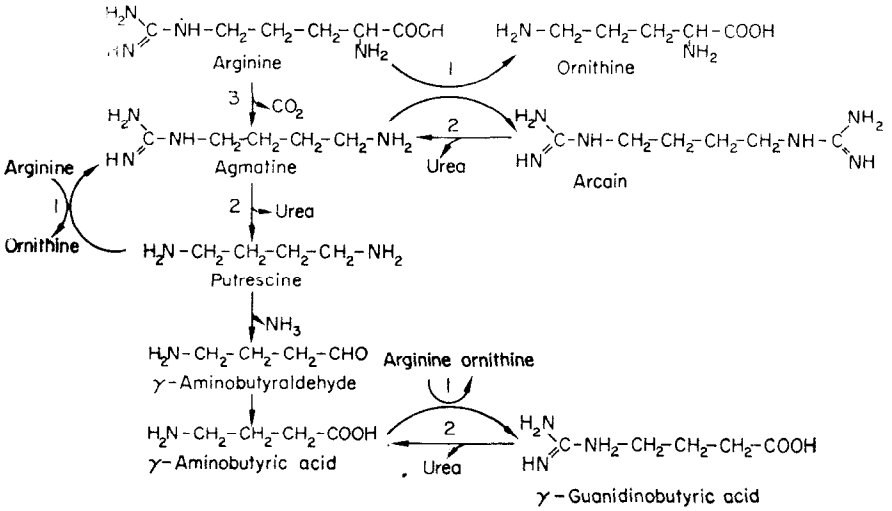

FIG. 2. SCHEMATIC REPRESENTATION OF ARGININE METABOLISM IN FRUIT-BODIES OF *Panus tigrinus*. (1) Transamidinase; (2) Ureohydrolase; (3) L-arginine carboxy-lyase.

[33] Y. ROBIN, C. AUDIT and M. LANDON, *Comp. Biochem. Physiol.* **22,** 787 (1967).
[34] J. B. WALKER and M. S. WALKER, *Biochem.* **6,** 3821 (1967).

EXPERIMENTAL

Cultivation of Panus tigrinus *and Feeding Technique*

P. tigrinus was cultivated under sterile and controlled conditions on a complex malt-agar medium as has been described.[11,36] Under the conditions employed (20–22°, 12 hr day), the mycelium fruited after 21–24 days. Fruit-bodies from the mycelium were fed through the stipe with one of the following radioactive compounds: L-arginine-U-^{14}C (UVVVR, Prague); L-arginine-amidine-^{14}C-HCl (Radiochemical Centre, Amersham); putrescine-1,4-^{14}C-dihydrochloride (Radiochemical Centre, Amersham); DL-ornithine-2 ^{14}C-dihydrochloride (Calbiochem); agmatine-*U*-^{14}C-sulphate (prepared from L-arginine-*U*-^{14}C). For the *in vitro* experiments DL-canavanine-amidine-^{14}C-dihydrochloride (CEA, France) was additionally used.

Paper and Thin-Layer Chromatography, Radioactive Measurements

Extraction of plant material, paper chromatography, and radio-autography were carried out as previously described.[11,36] Mixtures containing arginine, agmatine, arcain, putrescine, and ornithine were separated by thin-layer chromatography on Al_2O_2-layers or by paper chromatography (Table 3).

TABLE 3. R_f- VALUES OF BASIC COMPOUNDS IN TWO DIFFERENT SOLVENT SYSTEMS SEPARATED BY THIN-LAYER OR PAPER CHROMATOGRAPHY

Compound	TLC Al_2O_3 a*	TLC Al_2O_3 b*	Paper a*	Paper b*
Arginine	0·09	0·40	0·50	0·31
Agmatine	0·29	0·85	0·09	0·42
Arcain	0·00	0·79	0·00	0·00
Putrescine	0·75	0·69	0·80	0·33
Ornithine	0·25	0·38	0·62	0·25

* Solvents: (a), MeOH–25% NH_3–$CHCl_3$ 2:1:2 (vol); (b), n-BuOH–HOAc–pyridine–H_2O 4:1:1:2 (vol).

Enzyme Preparations

Crude enzyme preparations exhibiting transamidinase and ureohydrolase activities were prepared by extracting acetone powder as has been previously reported.[11]

L-*Arginine Carboxy-lyase*

L-Arginine decarboxylase was prepared by homogenizing 6 g of freshly harvested fruit-bodies of *P. tigrinus* with 3·0 ml 0·1 M Na-acetate buffer, pH 4·6, and 1·0 ml mercaptoethanol. Extraction was performed at 2° for 30 min. The suspension was centrifuged in the cold at 12,000 *g* for 60 min. The clear supernatant was dialysed against 0·05 M acetate buffer, pH 4·6, at 2° for 12 hr with constant stirring.

Putrescine Oxidase and γ-Aminobutyraldehyde Dehydrogenase

100 g of freshly harvested fruit-bodies of *P. tigrinus* were extracted by homogenizing with 40 ml 0·2 M phosphate buffer, pH 7·5, at 2°. After 30 min the suspension was centrifuged at 12,000 *g* for 60 min. The clear supernatant was dialysed against 0·06 M phosphate buffer, pH 7·5 and the dialysate was used as a crude enzyme preparation.

Enzyme Assays

(a) L-*Arginine carboxy-lyase*. The assays were carried out in a Warburg apparatus at 37°. The incubation mixture contained in a total of 3·0 ml: L-arginine, 4·0 μmoles, 0·2 M Na-acetate buffer, pH 5·2, and the resp. dialysed enzyme solution.

(b) *Putrescine oxidizing enzyme*. Determined by ammonia liberation in a coupled optical test with L-glutamate dehydrogenase. Decrease in extinction at 366 nm was followed up with time. The incubation mixture contained (μ moles): putrescine-di-HCl, 4·4, NADH–Na_2, 19·0, α-oxoglutarate, 0·57, phosphate buffer,

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[36] D. SCHLEE and H. REINBOTHE, *Phytochem.* **4**, 311 (1965).

pH 7·5, commercial glutamate dehydrogenase, 50 μl and resp. enzyme solution in a total volume of 3·0 ml. The reaction was initiated by adding the substrate. The oxygen uptake was determined in a Warburg apparatus at 30°. The mixture contained: putrescine–di-HCl, 5 μmoles; commercial catalase, 0·5 mg; phosphate buffer pH 7·5, and enzyme solution in a total of 3·0 ml. The central well of the Warburg vessels contained 20% KOH, 0·2 ml.

(c) *γ-Aminobutyraldehyde dehydrogenase.* The activity was determined in an optical test. The increase in extinction at 366 nm was followed with time. The reaction mixture contained: Δ^1-pyrroline, *0·6 μmoles, mercaptoethanol, 10·0 μmoles, NAD^+, 1·0 μmole, tris-HCl buffer, pH 7·8, and enzyme solution in a total volume of 2·0 ml.

Isolations and Chemical Preparations

Arcain was prepared as has been described.[12] Agmatine was isolated according to Smith[37] and crystallized as the sulphate (melting point of agmatine sulphate 228–230°). Agmatine-U-^{14}C was prepared from L-arginine-U-^{14}C by incubation with commercial L-arginine carboxy-lyase (*E. coli* acetone powder, Sigma). The reaction mixture contained in a total volume of 3·4 ml: 10·0 μmoles L-arginine-U-^{14}C (100 μc), 2·0 μmoles $MgSO_4$, 0·06 μmoles pyridoxal phosphate, 4·0 mg acetone powder from *E. coli* as enzyme source, and 640 μmoles tris-NaOH–maleate buffer, pH 5·2. Incubation was carried out at 37° for 2 hr. The reaction was stopped by heating at 100°. Agmatine-U-^{14}C was isolated after a slightly modified procedure according to Smith.[37] By means of preparative TLC, agmatine-^{14}C was purified, and crystallized as the sulphate with a specific activity of 0·112 mc/mmole.

Quantitative Determinations

Agmatine and arcain were estimated according to the procedure of Akamatsu and Watanabe.[38] Protein was estimated according to Lowry *et al.*[39] CO_2-liberation and O_2-consumption measurements were performed using the Warburg technique. Ammonia formation from putrescine was estimated in a coupled optical test with the aid of commercial L-glutamate dehydrogenase (Boehringer) according to the reaction: α-ketoglutarate + NH_3 + NADH + H^+ → L-glutamate + NAD + H_2O.

The decrease in extinction at 366 nm was followed. Estimation of γ-aminobutyraldehyde dehydrogenase activity was also based on the extinction measurements at 366 nm.

* Δ^1-Pyrroline was kindly given by Dr. L. Macholán, Brno, Czechoslovakia.

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