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UREA METABOLISM IN BASIDIOMYCETES—II
FORMATION OF γ-GUANIDOBUTYRIC ACID IN FRUIT BODIES OF *LYCOPERDON**

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Abstract—In experiments in which [guanido-^{14}C]-arginine was fed to fruit bodies of *Lycoperdon* spp., a high percentage of radiocarbon was incorporated into α-keto-δ-guanidovaleric and γ-guanidobutyric acids. A paper chromatographic survey checked the occurrence of γ-guanidobutyrate in puffballs of *Lycoperdon maximum*, *L. molle*, *L. perlatum* and *L. pyriforme*. The apparent route of γ-guanidobutyric acid formation from arginine is the oxidative pathway via α-keto-δ-guanidovaleric acid. From acetone dried puffballs of *L. perlatum* and *L. pyriforme* an enzyme was prepared that, in the presence of catalase, catalyses an oxidative deamination of L-(+)-arginine to α-keto-δ-guanidovalerianate. Without added catalase the keto compound is partially further oxidized to γ-guanidobutyric acid, obviously by means of hydrogen peroxide arising in the reaction. In fruit bodies of *Lycoperdon pyriforme* and *Agaricus bisporus* enzymes are present decomposing L-(+)-arginine and γ-guanidobutyric acid with formation of urea. Arginase could be extracted by means of diluted $MnSO_4$ solution. The γ-guanidobutyrate metabolizing system is under investigation.

INTRODUCTION

IN GENERAL, puffballs of *Lycoperdon* spp. contain very high amounts of urea. Estimates of urea content in *L. umbrinum*[1] have indicated values up to 8·7% of total fruiting body dry weight, representing 37% of the total nitrogen and 72% of the soluble N, corresponding to a concentration in the press-juice of about 0·15 M. The physiological function and ultimate fate of these large quantities of urea are not known. The biochemical events of urea formation now have been studied using radioactive compounds and purified enzymes.[1,2]

Urea synthesis along well-known lines of aerobic purine degradation has been established.[1,3] However, *de-novo* synthesis of ureides or purine precursors, respectively, is too unimportant to explain the high rate of urea formation and accumulation in fruit bodies of *Lycoperdon* and *Agaricus*. There is an analogous situation concerning pyrimidines. [2-^{14}C]-Uracil is very readily converted to [^{14}C]-urea. The reaction path is unknown. However, in experiments using [4-^{14}C]-aspartic acid *de-novo* synthesis of pyrimidines is unimportant (unpublished data).

From our isotopic experiments it is highly probable that urea biosynthesis in higher basidiomycetes mainly proceeds along the reactions of the Krebs–Henseleit ornithine cycle.[4] The mushroom carbamylation system has been partially purified by Levenberg.[2] Carbamyl phosphate synthesis in basidiomycete tissues employs the amide-N of L-glutamine as a

* Part I was published in *Flora* **152**, 423 (1962).

[1] H. REINBOTHE und B. TSCHIERSCH, *Flora* **152**, 423 (1962).
[2] B. LEVENBERG, *J. Biol. Chem.* **237**, 2590 (1962).
[3] A. BRUNEL, *Le métabolism de l'azote d'origine purique chez les champignons*. Thèse Doct. Sc. Nat., Paris 1936.
[4] H. A. KREBS und K. HENSELEIT, *Z. Physiol. Chem.* **210**, 33 (1932).

specific source of the carbamyl-nitrogen of citrulline. Ornithine transcarbamylase and arginase, as well as an enzyme that catalyses arginine formation from citrulline and L-aspartate in the presence of ATP and Mg^{2+} have been described from higher fungi.[5–7]

EXPERIMENTS AND RESULTS

In experiments in which arginine specifically labelled with carbon-14 in the guanido carbon was fed to puffballs of several *Lycoperdon* spp., a high proportion of radiocarbon was incorporated into two unknown guanido compounds (G_1, G_2) besides urea[1] (Table 1). Subsequent tests indicated that the radioactive arginine metabolites are α-keto-δ-guanidovaleric acid (G_1) and γ-guanidobutyric acid (G_2).

A paper chromatographic survey of the 70% ethanol extracts of puffballs of *Lycoperdon maximum*, *L. molle*, *L. perlatum*, and *L. pyriforme* revealed the occurrence of γ-guanidobutyrate in all of them. The keto compound (G_1) was detectable only after arginine had been fed.

TABLE 1. METABOLISM OF D,L-[GUANIDO-^{14}C]-ARGININE IN FRUIT BODIES OF *Lycoperdon* spp. PERCENT DISTRIBUTION OF RADIOACTIVITY IN COMPONENTS OF THE 70% ETHANOL EXTRACT

Species	Time (hr)	% Radioactivity in			
		Arginine	G_1*	G_2†	Urea
L. molle	8	65	5	11	17
L. perlatum	2	84	6	4	3
	10	58	10	19	9
L. pyriforme	8	66	0	10	22

* G_1 = α-keto-δ-guanidovaleric acid.
† G_2 = γ-guanidobutyric acid.

In puffballs of *Lycoperdon perlatum* γ-guanidobutyric acid labelling from $^{14}CO_2$ was only weak, whereas [U-^{14}C]-arginine was preferentially transformed to [^{14}C]-urea and [^{14}C]-γ-guanidobutyric acid.

Buffer extracts made from acetone powders of *L. perlatum* and *L. pyriforme* were fractionated by two ammonium sulphate precipitations (0–63%) and finally dialysed to remove all traces of the substrate. Incubation of L-(+)-arginine with this enzyme system resulted in the formation of α-keto-δ-guanidovaleric and γ-guanidobutyric acids. In a typical experiment the reaction mixture contained: enzyme solution (17·6 mg protein), 0·4 ml; L-arginine-HCl (2·5 μmoles), 0·1 ml; m/15 phosphate buffer, pH 7·3, 0·3 ml, in a total volume of 0·8 ml. The mixture was incubated at 34° and aliquots (40 μl) were withdrawn after 1, 2, 4, 6 and 24 hr for paper chromatographic analysis. Guanidine derivatives were located by the Sakaguchi spray reagent.

In experiments with carbon-14 labelled arginine, D,L-[guanido-^{14}C]-arginine-HCl (1·1 mc/mM; 1·2 μc)* was incubated with the enzyme in the presence of crystalline beef liver

* The L-isomer was not available for these experiments.

[5] P. P. COHEN and G. W. BROWN, Jr., in *Comparative Biochemistry*, Ed. M. FLORKIN and H. S. MASON, Vol. II, p. 161, New York (1960).
[6] S. YAMAMOTO, A. ERITATE and T. MIWA, *Bot. Mag.* (Tokyo) 66, 234 (1953).
[7] S. RATNER, *Advances in Enzymol.* 15, 319 (1954).

catalase and labelled α-keto-δ-guanidovalerate (G_1) accumulated in the reaction mixture as revealed by the radioautograph after two-dimensional paper chromatography (Fig 1, I). Without added catalase the keto compound was partially further degraded to γ-guanidobutyric acid. Presumably, our crude enzyme preparation was contaminated with catalase. In fresh dialysed preparations [^{14}C]-α-keto-δ-guanidovaleric acid was predominantly formed from [guanido-^{14}C]-arginine, whereas in enzyme solutions kept in the freezer over several weeks the additional formation of [^{14}C]-γ-guanidobutyric acid and two unknown labelled compounds was observed in analogous incubation experiments, these obviously being further oxidation products of the keto acid (Fig. 1).

Lycoperdon pyriforme enzyme was incubated at 34° with or without L-(+)-arginine-HCl in the presence of catalase. The complete reaction mixture contained: enzyme solution, 0·5 ml; m/15 phosphate buffer, pH 7·3, 0·2 ml; catalase solution (10 μmoles), 0·2 ml; and L-arginine-HCl (1·27 μmoles), 0·2 ml. Oxygen consumption was estimated using the Warburg

TABLE 2. *Agaricus bisporus*. FRUITING BODIES WITHOUT STIPE. UREA FORMATION IN HALF CAPS RELATED TO THE CONTROL HALF FROM ADDED GUANIDINE COMPOUNDS

Feeding*	μmoles/4 ml applied	μg urea/g fresh weight	%	μg urea/g dry weight	%
Water	—	1137	100	14910	100
γ-GBA	20	1312	115	27515	117
Water	—	831	100	9878	100
γ-GBA	40	1277	154	14722	150
Water	—	605	100	6900	100
γ-GB-NH_2	20	619	102	7143	103
Water	—	878	100		
Guanidine sulphate	20	875	101		
Water	—	866	100	7190	100
L-Arginine	20	1568	181	13850	179

* γ-GBA = γ-guanidobutyric acid; γ-GB-NH_2 = γ-guanidobutyric acid amide.

technique in intervals of 10 min up to 120 min. Using the microdiffusion procedure of Conway, ammonia evaluation was determined in analogous reaction mixtures, except that substrate concentrations were increased about 25-fold. The paper chromatographic examination revealed the appearence of α-keto-δ-guanidovaleric acid, the formation of which is correlated to oxygen uptake and ammonia evaluation. Surprisingly, added FAD (10^{-4} M) had no effect either on oxygen uptake or on ammonia formation from arginine. The fungal enzyme behaved quite similarly to a crude buffer extract made from dried *Agkistrodon piscivorus* venom which is known to possess about 1 % of L-amino acid oxidase acting also on arginine.

Preliminary feeding experiments with several guanido compounds have shown that in fruiting bodies of *Lycoperdon pyriforme* and *Agaricus bisporus*, L-arginine and γ-guanidobutyric acid raised the urea level. Caps of *Agaricus* were divided into halves. One half was supplied with water (control), the other with one of the guanidine derivatives cited above (Table 2). After 24 hr the urea content of the caps was estimated, using commercial urease, following the microdiffusion procedure of Conway.[8] Arginine and γ-guanidobutyric acid were obviously degraded with the concomitant formation of urea, whereas γ-guanidobutyric

[8] E. J. CONWAY and E. O'MALLEY, *Biochem. J.* 36, 655 (1942).

acid amide and guanidine sulphate did not raise the urea level. Degradation of γ-guanidobutyrate in *Agaricus* caps was also established using [guanido-^{14}C]-γ-guanidobutyric acid.

There is some evidence that arginine and γ-guanidobutyric acid are decomposed by different enzymes in basidiomycete tissues. Fruit bodies of *L. pyriforme* and *A. bisporus* were extracted with diluted $MnSO_4$ solution (0·5%). The supernatant obtained after centrifugation at 6000 *g* for 30 min was capable of splitting L-arginine, but not γ-guanidobutyric acid. An acetone powder made from fruiting bodies of *A. bisporus* was homogenized with a mixture of acetone–water (1:1). The homogenate was centrifuged at 14,000 *g* for 15 min. Incubation with the supernatant of 5 μmoles L-arginine, and 5 μmoles γ-guanidobutyric acid, respectively, resulted in the formation of 0·75 μmoles resp. 1·25 μmoles urea. (The reaction was stopped before guanidine breakdown was completed.) Further investigations are however necessary to elucidate patterns of guanidine metabolism in basidiomycete tissues.

DISCUSSION

Evidence is presented that γ-guanidobutyric acid present in puffballs of *Lycoperdon* spp. is synthesized by an oxidative deamination of L-arginine. An oxidative pathway of γ-guanidobutyrate formation via α-keto-δ-guanidovaleric acid has been reported in marine invertebrates,[9] insects,[10] and a strain of gram-positive bacteria.[11] Some evidence for this mode of γ-guanidobutyrate synthesis also occurring in higher plants comes from the recent work of Barnes.[12] The labelling patterns he found in pine tissues fed uniformly labelled arginine, are more consistent with the hypothesis of the oxidative pathway than with a synthesis by transamidination.

Our enzyme preparations made from acetone powders of *L. perlatum* and *L. pyriforme* behaved quite similarly to a buffer extract made from dried *Agkistrodon piscivorus* venom. L-(+)-Arginine is oxidative deaminated to α-keto-δ-guanidovaleric acid which is further oxidized to γ-guanidobutyric acid obviously by means of hydrogen peroxide arising in the reaction. In the presence of added crystalline beef liver catalase the keto compound accumulates. Its formation is coupled to oxygen uptake and ammonia evolution.

Our enzyme preparation was free from any transamidinase activity with arginine as amidine donator. [2-^{14}C]-Ornithine was not incorporated into arginine. In trapping experiments with hydroxylamine acting as amidine acceptor in slow transamidination reactions, no hydroxyguanidine formation was found. However, in the presence of the carbonyl reagent α-keto-δ-guanidovaleric and γ-guanidobutyric acids were not detectable by radioautography. When [guanido-^{14}C]-arginine was incubated with the fungal enzyme in the presence of hydroxylamine an unknown carbon-14 labelled compound appeared, obviously being the product of a non-enzymic reaction of the keto acid with the carbonyl reagent. We therefore suppose that the fungal enzyme studied is an amino acid oxidase acting on L-arginine. Nothing is known about the substrate specificity of this enzyme. It seems unlikely that the investigated system is a component one. One could claim that α-keto-δ-guanidovaleric acid is formed by a transamination reaction involving L-arginine (as the added substrate) and traces of α-keto-glutaric or pyruvic acids possibly retained in the dialysed enzyme. Transamination could be followed by an oxidative deamination of resulting glutamic acid or L-alanine by means of a specific L-amino acid oxidase. The net effect would be an

[9] J. ROCHE, Ng. V.-THOAI et P. GLAHN, *Experientia* **8**, 428 (1952).
[10] I. GARCIA, M. TIXIER et J. ROCHE, *Compt. Rend. Soc. Biol.* **150**, 632 (1956); **151**, 1844 (1957).
[11] M. NAKADA, *Med. J. Osaka Univ.* **5**, 353 (1954).
[12] R. L. BARNES, *Nature* **193**, 781 (1962).

oxidative deamination of L-arginine. The keto compound involved as amino group acceptor in transamination would be continuously regenerated thus acting in catalytic amounts. γ-Guanidobutyric acid formation might be understood in the same manner as in the case of L-arginine oxidation as a non-enzymic reaction with hydrogen peroxide. But incubation of the enzyme with L-glutamic acid, L-aspartic acid, or L-alanine resulted in no ammonia evolution as in the case of L-arginine. Further studies have to be done to estimate substrate specificity of fungal L-amino acid oxidase.

An enzyme catalysing the oxidative deamination of some 10 amino acids was found for the first time in *Proteus vulgaris*.[13] Highly active L-amino acid oxidases occur in tissues of animals showing the uricotelic type of nitrogen excretion. Snake venoms are especially rich sources of such enzymes.[14] L-Amino acid oxidase from moccasin (*Agkistrodon piscivorus*) venom has been highly purified.[15] The enzyme from rattlesnake (*Crotalus adamanteus*) venom has been isolated and obtained for the first time in crystalline form.[16] This enzyme is a flavoprotein containing two molecules of FAD per molecule of protein. The substrate specificity of the amino acid oxidases found in nature varies with respect to individual amino acids. But all are capable of catalysing the oxidative deamination of a large number of L-amino acids according to the general scheme: shown in Fig. 2. In the absence of catalase the keto acid is further oxidatively degraded.

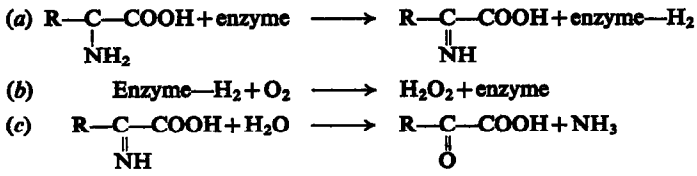

FIG. 2. ACTION OF L-AMINO ACID OXIDASES.

γ-Guanidobutyric acid formation in puffballs of *Lycoperdon* might be correlated to urea formation. A γ-guanidobutyrate pathway of urea synthesis from arginine might be a method of by-passing the arginase-catalysed urea formation. But there is little evidence that such a reaction path is of any importance in fruit bodies of *L. pyriforme* and *A. bisporus*, for arginase is present.

Surprisingly, $^{14}CO_2$ is only weakly introduced in γ-guanidobutyric acid, whereas radiocarbon from exogenously supplied [guanido-^{14}C]-arginine and [U-^{14}C]-arginine is very readily incorporated into γ-guanidobutyrate. These findings may reflect the different behaviour of endogenously formed and exogenously supplied arginine in fungal metabolism. An excess of arginine might be transferred to the "guanidine pool" thus representing a regulation mechanism caused by compartmentalization, some mode of substrate inhibitions or different substrate affinities of arginine metabolizing enzymes. This problem is under investigation.

EXPERIMENTAL

1. *Paper chromatography and radioactive measurements.* Paper chromatographic analysis was carried out on Schleicher and Schüll 2043b paper using the two-dimensional ascending technique. The chromatograms of the radioactive experiments were radioautographed

[13] P. K. STUMPF and D. E. GREEN, *J. Biol. Chem.* **153**, 387 (1944).
[14] E. A. ZELLER, *Advances in Enzymol.* **8**, 458 (1948).
[15] T. S. SINGER and E. B. KEARNEY, *Arch. Biochem.* **27**, 348 (1950); **29**, 190 (1950).
[16] D. WELLNER and A. MEISTER, *Abstract, 4th International Congress of Biochemistry*, Vienna, Austria (1958).

using Diavidox X-ray film (Fotochemische Werke Berlin) for periods depending on the amount of radioactivity applied (Fig. 1). The radioactivity of a spot on the chromatogram corresponding to the darkened area of the radioautograph was determined by counting on the paper by means of a thin-end-window Geiger–Müller tube, and calculated as a percentage of the total radioactivity of all the radioactive areas detectable by radioautography.

2. *Identification of G_1 and G_2.* The identification of the unknowns (G_1 and G_2) formed in puffballs of *Lycoperdon* spp. fed with [guanido-^{14}C]-arginine was carried out as follows: (*a*) Tissue extracts were treated according to the ion-exchange procedure of Plaisted[17] and the unknown G_1 was partially converted to G_2, both being eluted from Dowex 50 (H^+) resin with the acidic and neutral amino acids. Both compounds are detectable on papers with alkaline ferricyanide–nitroprusside reagent or a Sakaguchi spray.[18] G_1 also responded to the picric acid reagent (Jaffé) and 2,4-dinitrophenylhydrazine.[18] (*b*) The unknown G_2 was chromatographically identical with authentic γ-guanidobutyric acid in 6 different solvents. G_1 behaves identically to an authentic sample of α-keto-δ-guanidovaleric acid obtained by incubating dried *Agkistrodon piscivorus* venom (used as a crude L-amino acid oxidase preparation) with L-arginine in the presence of crystalline beef liver catalase. In experiments with [guanido-^{14}C]-arginine the positions of radioactive spots G_1 and G_2 (as revealed by the radioautograph) coincided exactly with the colour produced from the two authentic compounds added as carriers by the Sakaguchi reagent. (*c*) [^{14}C]-γ-Guanidobutyric acid was eluted from papers and subjected to alkaline hydrolysis following the procedure of Bell.[19] The reaction mixture was passed through a column of Dowex 50 (NH_4^+) resin. After evaporation to a small volume, the effluent was chromatographed as described above. [^{14}C]-Urea, γ-aminobutyric acid and an unidentified carbon-14 compound (presumably γ-ureidobutyric acid) were detected after developing the papers with a ninhydrin spray and Ehrlich's reagent. γ-Aminobutyric acid was chromatographically identified, [^{14}C]-urea was degraded to ammonia and $^{14}CO_2$ with commercial urease. (*d*) α-Keto-δ-guanidovaleric acid (G_1) was decarboxylated to γ-guanidobutyric acid by means of hydrogen peroxide. The keto compound was also eluted from papers and subjected to a non-enzymic transamination with pyridoxamine at 100° in the presence[20] of Al^{3+}. Small amounts of arginine were formed and chromatographically identified.

3. *Preparations.* [U-^{14}C]-arginine was biologically synthesized by Dr. B. Tschiersch in this laboratory. Leaves of *Nicotiana rustica* photosynthesized in the presence of $^{14}CO_2$ over several weeks. Isolated protein was subjected to an acidic hydrolysis. [^{14}C]-arginine was isolated from the amino acid mixture by means of ion exchange and paper chromatographic methods.

D,L-[guanido-^{14}C]-arginine was incubated with the fungal arginine oxidizing enzyme. The [^{14}C]-α-keto-δ-guanidovaleric acid obtained (besides labelled γ-guanidobutyric acid) was converted by means of hydrogen peroxide to γ-guanidobutyrate and the latter isolated using chromatographic techniques.

Enzyme preparation: Acetone powders were prepared from puffballs of *Lycoperdon perlatum* and *L. pyriforme* by homogenizing 25-g batches of frozen fruiting bodies with 500 ml of cold acetone, allowing the residue to settle, decanting, and again homogenizing the precipitate with fresh acetone. The precipitate was harvested by centrifugation, and the

[17] P. H. PLAISTED, *Contr. Boyce Thompson Inst.* **19**, 231 (1958).
[18] I. M. HAIS und K. MACEK (Hrsg.), *Handbuch der Papierchromatographie*, Vol. I, Jena (1958).
[19] E. A. BELL, *Biochem. J.* **85**, 91 (1962).
[20] A. MEISTER, *J. Biol. Chem.* **206**, 577 (1954).

residual acetone was removed *in vacuo*. The enzyme was extracted by homogenizing 2·6 g of acetone powder with 42 ml m/15 phosphate buffer, pH 7·5, containing 260 mg of EDTA. The mixture was centrifuged at 14,000 *g* for 15 min. A saturated solution of ammonium sulphate was added slowly to the supernatant to a final concentration of 63% saturation. After 15 min the mixture was again centrifuged at 14,000 *g* for 15 min. The precipitate was dissolved in 9 ml m/15 phosphate buffer, pH 7·5, containing 50 mg EDTA. A saturated ammonium sulphate solution was added dropwise as before. The precipitate obtained by centrifugation at 14,000 *g* for 15 min was redissolved in a minimal volume of m/15 phosphate buffer, pH 7·5, plus EDTA. The solution was dialysed for 18 hr at 4° against phosphate buffer (m/150, pH 7·5). The dialysed enzyme solution, which was free from Sakaguchi-positive compounds, was stored in the freezer. The final protein concentration (estimated following Lowry) was 44 mg/ml.

4. *Quantitative determinations.* Urea and ammonia were determined using the micro-diffusion procedure of Conway[8] using commercial urease. Oxygen consumption was followed by means of the Warburg technique.

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