METABOLISM OF CITRULLINE AND ORNITHINE IN MUNG BEAN MITOCHONDRIA[1]

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The occurrence of appreciable quantities of citrulline (31) and ornithine (26) in some higher plants and recently the discovery of trace amounts of these amino acids in others, such as barley and wheat (8) has led to speculations about their metabolism. Little information is available on the interconversions of these amino acids in plant tissue. It is known that when ornithine-2-C^{14} is administered to barley and white clover plants, the C^{14} label appears in citrulline, arginine, proline and glutamic acid (3). Further, Krebs and Eggleston (11) have detected citrulline phosphorylase activity in homogenates of the beans *Phaseolus lunatus* and *P. vulgaris*, and the pea, *Pisum sativum*. Beyond this, conjecture has had to rely mainly on data from work with bacteria and mammalian tissues.

It is possible that the mechanism by which citrulline is synthesized in plants is similar to that in extracts of *Streptococcus faecalis* (7) and rat liver (4, 7) and involves the following reactions:

$$\text{ATP} + CO_2 + \text{ammonia} = \text{carbamyl phosphate} + \text{ADP}$$

$$\text{carbamyl phosphate} + \text{ornithine} = \text{citrulline} + \text{inorganic phosphate}$$

The liver carbamyl phosphate synthetase was found to have a requirement for N-acetylglutamate which was unnecessary for *S. faecalis* extracts (14). Reichard (20) purified ornithine carbamyl transferase from liver and found it identical with the citrulline phosphorylase enzyme of Krebs et al (12).

Two feasible pathways that may exist in higher plants for the biosynthesis of ornithine are suggested by studies of the synthesis of ornithine from glutamate in the microorganisms *Neurospora crassa* and *Escherichia coli*. In *N. crassa*, ornithine arises from the transamination of glutamic-γ-semialdehyde (28), whereas in *E. coli* synthesis proceeds via several N-acetylated derivatives (27). It remains possible, of course, that yet another mechanism occurs in higher plants.

The present study is concerned with the enzymatic synthesis of citrulline and ornithine by mung bean (*Phaseolus aureus*) mitochondria and the role of carbamyl phosphate in the conversion of ornithine and citrulline.

MATERIALS AND METHODS

Mung bean seeds were soaked for 3 hours in tap water and then planted in moistened vermiculite contained in wooden seed boxes. The boxes were covered with glass plates to keep the humidity maximal. The seedlings were grown in the dark at 31° C for 2 days. Mitochondria were prepared from washed seedlings by the technique of Millerd et al (17) except that the preparative medium used throughout consisted of 0.1 M phosphate buffer (KH_2PO_4) pH 7.4, 0.4 M sucrose and 0.01 M disodium ethylenediamine tetracetic acid (EDTA). Mitochondrial suspensions were incubated with experimental media at 30° C.

Carbon dioxide was measured manometrically in the Warburg apparatus. Ammonia was determined by distillation in Conway units, followed by Nesslerization and measurement of the color produced in a Unicam spectrophotometer, Model SP 500, at 410 mμ. Citrulline was estimated by the modified ninhydrin reaction of Vogel and Bonner (29). Glutamic acid was determined manometrically using the glutamic acid decarboxylase of *Clostridium welchii* (10). Glutamic-γ-semialdehyde was assayed by the method of Vogel and Davis (30) using the mutant strains of *E. coli*, 55-25 and 55-1.

To isolate citrulline, ornithine and glutamic acid, reaction mixtures were deproteinized with sufficient glacial acetic acid to give a final concentration of 10 % acid, and then warmed gently for a few minutes. The protein was centrifuged off at 5,000 × G for 5 minutes. Aliquots of the deproteinized solution were banded onto paper strips (Whatman no. 3 MM), 5 cm × 40 cm. These strips were subjected to paper electrophoresis in a Shandon vertical electrophoresis apparatus using 0.1 M potassium bicarbonate as the electrolyte and a potential of 200 volts. A satisfactory separation of ornithine from citrulline and glutamate from aspartate was achieved in 2 hours under these conditions. Following drying at room temperature, the amino acids were located by spraying the edges of the bands with 0.05 M citric acid dissolved in acetone, then overspraying with a ninhydrin solution (0.1 % w/v in 95 % ethanol) and heating at 80° C for 10 minutes. The desired amino acids were each eluted from the paper with 5 ml of distilled water and the eluates evaporated to dryness. To free the amino acids from any radioactive contaminants, one dimensional paper chromatograms were run using diisopropyl ether : 90 % formic acid (3 : 2) (11) for ornithine and citrulline, and the organic layer of *n*-butanol : acetic acid : water (4 : 1 : 5) for glutamic acid. The solvent, 80 % aqueous phenol was also occasionally used for identification purposes.

The stimulatory effect of arsenate on citrulline

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phosphorylase (12) was used to measure the C^{14} content of the carbamyl group of citrulline. Citrulline phosphorylase was prepared from mung bean seedlings homogenized in sodium arsenate solution and sufficient hydrochloric acid to give a final concentration of 0.1 M arsenate buffer, pH 6.7. This homogenate was stored for 16 hours at 4° C to lower the endogenous carbon dioxide production and then used in the standard Warburg apparatus, under anaerobic conditions to decarboxylate citrulline. The activity of the homogenate was of the same order as that of mitochondrial preparations. Control flasks containing no citrulline and experimental flasks containing citrulline were used in duplicate with either alkali or water in the center well. The reaction was allowed to proceed for 3 hours, when 2 *N* hydrochloric acid was tipped into the reaction mixture to dispel the trapped carbon dioxide and stop the reaction. The $C^{14}O_2$ absorbed in 0.2 ml of *N* sodium hydroxide in the center well was estimated and from the total carbon dioxide produced, as measured manometrically, the specific activity of the carbamyl carbon was calculated.

Radioactive glutamic acid was degraded as described by Handler and Anfinsen (5). Carbon 1 was liberated as carbon dioxide by the action of *Cl. welchii* and the γ-aminobutyric acid produced was degraded by the Schmidt reaction. Trimethylene diamine dipicrate was isolated and the radioactive content measured.

Ornithine was decarboxylated using the acid permanganate reaction of Strassman and Weinhouse (23) and the resulting $C^{14}O_2$ was assayed for radioactivity.

All radioactive samples were assayed as described by Moses (18) using a windowless gas flow (methane) proportional chamber connected to a scaler.

N-acetylglutamate was prepared according to Nicolet (19). Carbamyl phosphate was prepared and stored as the lithium salt (7) and when required, converted into the barium salt (25). C^{14}-labeled compounds were obtained from the Radiochemical Centre, Amersham, England. O.P.C. 45, a non-ionic detergent was a gift from Petrochemicals Ltd. (London).

Results

Synthesis of Ornithine: To determine the pathway of the conversion of Krebs cycle intermediates to glutamate and ornithine, the incorporation of acetate-1-C^{14} into these amino acids was studied. Mitochondria in the presence of 1.3 micromoles (μM) of acetate-1-C^{14} (2×10^6 cpm) and 10 μM of malate, and under the same experimental conditions as those in table I except that no amino acids were added, utilized 68 % of the initial acetate in 3 hours. Of this, 47 % was oxidized to carbon dioxide and 53 % was incorporated into mitochondrial substances. Malate was added to the experimental medium to supply ATP and oxalacetate (15) which are necessary for acetate oxidation (16). The oxidation of acetate-1-C^{14} indicates the presence of an acetate activating enzyme which has been previously demonstrated in crude extracts of mung bean seedlings by Millerd and Bonner (15). When mitochondria were incubated with acetate-1-C^{14}, malate and either glutamate or ornithine, 2 % of the acetate-1-C^{14} utilized was converted into glutamate and a smaller quantity into ornithine (table I). The low specific activity of ornithine, as compared with that of glutamate, indicates that ornithine is not so easily synthesized from α-ketoglutaric acid. The distribution of the C^{14} within the isolated amino acids is given in table I. Acetate-1-C^{14} was found to be incorporated into the carboxyl groups of glutamic acid, most of it being located in the γ-carboxyl group. This result implies that the tracer is incorporated by way of the Krebs cycle. Comparison of the C^{14} content of carbon 1 and of carbons 2 + 3 + 4 + 5 of glutamate and ornithine would seem to indicate derivation from a common carbon skeleton.

Studies were carried out on the conversion of glutamate-U-C^{14} (uniformly labelled) into ornithine by a system containing 0.1 μM of glutamate-U-C^{14} (100,000 cpm) and 20 μM of ornithine in the presence of a suspension of mitochondria. The mitochondria incorporated 8 % of the initial glutamate-U-C^{14} into ornithine. The addition of 0.001 M sodium arsenite

Table I

Distribution of C^{14} in Glutamate and Ornithine Following Incorporation of Acetate-1-C^{14} By Mung Bean Mitochondria

Amino Acid Added	Amino Acid at end of Experiment	Radioactivity incorporated	Specific activity	Percentage of total activity in carbon atoms		
	μM/flask	cpm	cpm/μM	1	2 + 3 + 4	5
Glutamate	44.2	28,370	642	9	0	88
Ornithine	48.1	3,120	65	13	87	

Complete system contained: 10 μM sodium 1-malate, 1.3 μM sodium acetate-1-C^{14} (2×10^6 cpm), 400 μM sucrose, 100 μM phosphate buffer pH 7.4, 10 μM EDTA, 20 μM $MgSO_4$, 1 μM ATP, 40 μg terramycin, 50 μM 1-amino acid and 1.0 ml of mitochondrial suspension. Total volume 2.0 ml. Temp, 30° C; gas phase, air; duration of experiment, 3 hours.

to the system, however, reduced the amount incorporated to 2 %. Mitochondria oxidized ornithine to a limited extent (an uptake of 1.1 microatoms of oxygen per flask in 30 minutes). It was known from previous work that mung bean mitochondria contain traces of α-ketoglutaric acid (2) and, after the oxidation of ornithine, the system was found on analysis to have formed small amounts of glutamic-γ-semialdehyde and glutamic acid. Arsenite, at a concentration of 0.001 M, inhibited the conversion of glutamic acid to α-ketoglutaric acid. The inhibition by arsenite of the conversion of ornithine into glutamic acid and of glutamate into ornithine is of particular interest in view of Strecker's (23) finding that the synthesis of glutamic-γ-semialdehyde from glutamate by whole cells of a proline auxotroph of *E. coli* can be inhibited by arsenite.

The inhibition of aldehyde dehydrogenase by arsenite was studied by Jakoby (6), who found that the arsenite inhibition involved thiol groups. In view of this, it seemed a reasonable possibility that the sensitivity of ornithine oxidation to arsenite was due to the presence of thiol groups. The possible formation of a thioester of the γ-carboxyl group of glutamic acid was therefore investigated by using hydroxylamine as a trapping agent and estimating γ-glutamohydroxamic acid (13); no glutamohydroxamic acid was formed. Mitochondria did not phosphorylate glutamic acid. Glutamohydroxamic acid was only formed when glutamic acid, ATP, cysteine, magnesium sulphate, hydroxylamine were incubated with mitochondria. This is the condition for the assay of glutamine synthetase which has been shown to occur in bean mitochondria (32). Attempts to demonstrate the formation of glutamic-γ-semialdehyde from glutamic acid by mitochondria were unsuccessful. The conversion of ornithine to glutamic-γ-semialdehyde by the δ-ornithine-glutamate transaminase was demonstrated, however, in these mitochondria. It was found that 0.5 μM of glutamic-γ-semialdehyde was formed in 1 hour from 20 μM of ornithine and 20 μM of α-ketoglutarate at pH 7.4 by a heavy suspension of mitochondria. Glutamic acid, the other product of the reaction, was identified by one-dimensional paper chromatography. The presence of δ-ornithine-glutamate transaminase has recently been demonstrated in crude extracts of spinach leaves by Scher and Vogel (22).

INTERCONVERSION OF CITRULLINE AND ORNITHINE: Arsenolysis of citrulline which is inhibited by phosphate, is characteristic of the citrulline phosphorylase system (12). Mung bean mitochondria washed free from phosphate with 0.4 M sucrose solution were found to carry on a slow anaerobic decarboxylation of citrulline in the presence of 0.1 M sodium arsenate, pH 6.7, releasing 18 microliters of carbon dioxide per hour per flask. The presence of 0.05 M phosphate buffer, pH 6.7, inhibited the arsenolysis by 85 %. Arsenolysis was found to produce 1 molecule of carbon dioxide and 1 molecule of ammonia from 1 molecule of citrulline. Ornithine was formed and identified by one-dimensional paper chromatography. Due to the low activity of the system, citrulline phosphorylase could not be directly demonstrated in these mitochondria by measuring the carbon dioxide released when citrulline was incubated with 0.1 M phosphate buffer.

However, 0.4 μM of citrulline were found to be synthesized in 1 hour from 20 μM of carbamyl phosphate and 20 μM of ornithine by mung bean mitochondria suspended in 0.05 phosphate buffer, pH 7.4. Under these conditions the phosphorolysis of citrulline is completely inhibited by the presence of ornithine. Citrulline was further identified by one-dimensional chromatography with 3 different solvents. This evidence suggest the presence of citrulline phosphorylase.

To study the synthesis of carbamyl phosphate by mitochondria, the following system was incubated for 1 hour at 30° C: 50 μM phosphate buffer pH 7.4, 10 μM ammonium chloride, 10 μM ATP, 10 μM N-acetylglutamate, 10 μM magnesium sulphate, 10 μM $NaHC^{14}O_3$ (200,000 cpm), 200 μM sucrose and 0.5 ml. of mitochondrial suspension to give a final volume of 1.0 ml. The control system contained no ammonium chloride. At the end of the experiment the reaction mixture was cooled to 0° C and the rest of the manipulations were carried out at 0° C. Twenty μM of lithium carbamyl phosphate were added to the reaction mixture and immediately followed by sufficient cold, concentrated perchloric acid to bring the pH of the system to 2.0. Carbamyl phosphate was isolated

TABLE II

DEGRADATION OF CARBAMYL PHOSPHATE BY MUNG BEAN MITOCHONDRIA

EXPERIMENT	ADDITIONS TO SYSTEM	CO_2 μL/HR/FLASK
1	None	6.1
2	$MgSO_4$	11.8
3	Glucose, ATP, Hexokinase	20.6
4	$MgSO_4$, Hexokinase, Glucose, ATP	29.8
5	$MgSO_4$, Hexokinase, Glucose, ATP, N-acetylglutamate	46.1
6	Expt 4, O.P.C. 45	63.2
7	Expt 5, O.P.C. 45	80.6

Each flask contained: 0.3 ml of mitochondrial suspension, 10 μM lithium carbamyl phosphate, 100 μM phosphate buffer pH 6.1, 400 μM sucrose and 10 μM EDTA. The additions to reaction were 10 μM $MgSO_4$, 50 μM glucose, 1 mg hexokinase, 4 μM ATP, 10 μM N-acetyl-1-glutamate and 0.1 % (v/v) O.P.C. 45. Total volume 2.0 ml. Gas phase, N_2; Temp, 30° C. Additions were adjusted to pH 6.1. All results corrected for control system which contained boiled mitochondria, lithium carbamyl phosphate and $MgSO_4$.

from the deproteinized mixture as an ethanol-insoluble barium salt (25). This barium salt was dissolved in water, decomposed with normal hydrochloric acid in the Warburg apparatus and the liberated $C^{14}O_2$ was trapped and assayed. The barium salt obtained was tentatively identified as barium carbamyl phosphate by the method of isolation and by the rate of release of $C^{14}O_2$ compared with CO_2 produced from authentic barium carbamyl phosphate under acid conditions. It was possible under these conditions to obtain the synthesis of 0.1 μM of radioactive carbamyl phosphate.

The degradation of carbamyl phosphate by mung bean mitochondria could be more easily demonstrated than its synthesis (table II). Products of the reaction were ammonia and carbon dioxide. Increased decarboxylation at pH 6.1 was obtained with magnesium sulphate, the hexokinase system and N-acetylglutamate. Hexokinase and glucose convert ATP into ADP which reacts with carbamyl phosphate to form ATP, ammonia and carbon dioxide (4, 7). The inclusion of a non-ionic detergent, O.P.C. 45, which is an effective agent for disrupting the mitochondrial membrane (2), enhanced the degradation of carbamyl phosphate.

TABLE III

INCORPORATION OF $NaHC^{14}O_3$ INTO CITRULLINE BY MUNG BEAN MITOCHONDRIA

ADDITIONS TO REACTION SYSTEM	RADIOACTIVITY IN CITRULLINE (CPM)
None	5,800
N-acetylglutamate	6,080
N-acetylglutamate and Carbamyl phosphate	4,000
N-acetylglutamate with boiled Mitochondria	0

Each flask contained: 0.3 ml of mitochondrial suspension, 40 μM sodium succinate, 2 μM ATP, 10 μM $MgSO_4$, 20 μM l-ornithine, 20 μM NH_4Cl, 10 μM $NaHC^{14}O_3$ (200,000 cpm), 200 μM sucrose, 5 μM EDTA, 50 μM phosphate buffer pH 7.4. Additions to flasks, 5 μM N-acetyl-l-glutamate and 2 μM lithium carbamyl phosphate. Total volume 1.0 ml. Temp, 30° C; gas phase, air. At end of 1 hour, 10 μM of carrier l-citrulline added, immediately followed by deproteinization of the reaction systems.

That citrulline is synthesized by mitochondria at pH 7.4 from $NaHC^{14}O_3$, ammonium chloride, ornithine and a source of ATP is shown in table III. The presence of N-acetylglutamate had no effect on the synthesis of citrulline, but the isotopic dilution effect of added carbamyl phosphate indicates that carbamyl phosphate is an intermediate in the synthesis of citrulline. The citrulline isolated from this experiment showed 95% of C^{14} to be located in the carbamyl carbon.

DISCUSSION

It has been demonstrated by this series of experiments that acetate-1-C^{14} can be oxidized by mung bean mitochondria. If the assumption is made that the oxidation occurs via acetyl-CoA, which then enters the Krebs cycle, it would be expected that the labelling patterns produced in the intermediates of the cycle would be characteristic of acetate-1-C^{14}. After several turns of the cycle α-ketoglutarate would contain C^{14} in carbons 1 and 5 (21), and as these mitochondria contain glutamate dehydrogenase (2), the labeling in glutamic acid would also be located in carbons 1 and 5. The labeling pattern in glutamate which was in fact obtained, is consistent with the operation of a Krebs cycle in these mitochondria, if the presence of malate is taken into account. Malate acts as an isotopic diluent of malate-C^{14} so that the percentage of C^{14} in carbon 1 is decreased from the theoretical value of 33 % which would be achieved in its absence. The presence of a Krebs cycle was also indicated by the work of Millerd (15) on the oxidation of pyruvate by mung bean mitochondria. A similar isotopic pattern in glutamate was found by Bilinski and McConnell (1) when growing wheat plants were inoculated with acetate-1-C^{14}.

The presence of δ-ornithine-glutamate transaminase, the incorporation of glutamate-U-C^{14} into ornithine, and the inhibition of both this reaction and the oxidation of ornithine by arsenite, favors the synthesis of ornithine by the pathway occurring in *N. crassa* (28). That is, the synthesis of ornithine from glutamate in mung bean mitochondria involves the intermediate formation of glutamic-γ-semialdehyde, rather than, as occurs in *E. coli* (27), of acetylated derivatives of glutamate and ornithine. While the similarity of the distribution of C^{14} in glutamate and ornithine cannot be counted as direct support for the presence of this pathway, it is, however, compatible with its occurrence.

The synthesis of citrulline from ornithine, carbon dioxide and ammonia in mung bean mitochondria involves a carbamyl phosphate synthetase and citrulline phosphorylase, as was found to be the case with rat liver mitochondria and *S. faecalis* extracts (7). The behavior of N-acetylglutamate in mung bean mitochondria is different from that in either of these 2 systems since it activates carbamyl phosphate degradation while having no effect on citrulline synthesis.

SUMMARY

1. C^{14} from acetate-1-C^{14} is incorporated into carbons 1 and 5 of glutamate by mung bean mitochondria. The labeling pattern is consistent with the operation of the Krebs cycle.

2. Ornithine is synthesized from glutamate and ornithine-glutamate transaminase is present.

3. Citrulline is synthesized from carbon dioxide, ammonia and ornithine with carbamyl phosphate acting as the carbamyl group donor.

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