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PRODUCTS OF ARGININE CATABOLISM IN GROWING CELLS OF SUGARCANE*

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Abstract—On incubating sugarcane cells of variety H 50-7209 in a synthetic liquid medium in the presence of ^{14}C-arginine, a substantial proportion of the label was found in *N*-carbamylputrescine, while a much smaller proportion appeared in guanidine. Large amounts of ^{14}C-arginine were found in the ethanol-soluble fraction of the cells, and labelled arginine, proline and glutamic acid were incorporated in the proteins. The *N*-carbamylputrescine which began to accumulate within 10 min after the addition of ^{14}C-arginine to the cell cultures was not excreted, nor did it appear to be further metabolized by these cells. Evidence was found that *N*-carbamylputrescine is formed more readily from citrulline than from arginine in sugarcane cells.

INTRODUCTION

AGMATINE, *N*-carbamylputrescine, and putrescine are produced in higher plants from arginine under both normal and abnormal conditions of growth.[1-3] Smith[4,5] demonstrated significant increases in arginine decarboxylase and *N*-carbamylputrescine amido hydrolase in barley seedlings grown under conditions of potassium deficiency. Agmatine and putrescine concentrations were elevated under potassium stress, not only in barley but in numerous other plant species.[4] Smith and Garraway[2] fed agmatine to barley seedlings and identified *N*-carbamylputrescine as an intermediate of putrescine formation. In tobacco plants the catabolism of arginine via putrescine appears to provide an alternate route for the formation of the pyrrolidine ring of nicotine.[6]

Arginine at a concentration of 50 ppm has been shown to enhance the growth of sugarcane plants when fed through the roots of the intact plant in nutrient solution.[7] A similar response of increased growth rate can be demonstrated in sugarcane cell cultures when arginine is supplied as a constituent of the growth medium at a level of 60 ppm.[8]

To obtain more knowledge about the metabolic fate of arginine in sugarcane, we studied the role of this amino acid in sugarcane cell cultures. Sugarcane cells supplied exogenously with ^{14}C-arginine were found to convert an appreciable amount of arginine to *N*-carbamylputrescine; the present report deals with this phase of our study.

* Part II in the series "Arginine in Growing Cells of Sugarcane".

[1] L. FOWDEN, *Ann. Rev. Plant Physiol.* **18**, 85 (1967).
[2] T. A. SMITH and J. L. GARRAWAY, *Phytochem.* **3**, 23 (1964).
[3] T. A. SMITH and F. J. RICHARDS, *Biochem. J.* **84**, 292 (1962).
[4] T. A. SMITH, *Phytochem.* **2**, 241 (1963).
[5] T. A. SMITH, *Phytochem.* **4**, 599 (1965).
[6] D. YOSHIDA and T. MITAKE, *Plant Cell Physiol.* **7**, 301 (1966).
[7] L. G. NICKELL and H. P. KORTSCHAK, *Hawaiian Planter's Record* **57**, 230 (1964).
[8] L. G. NICKELL and A. MARETZKI, *Physiol. Plantarum*, in press.

RESULTS

Sugarcane cells were partially depleted of arginine by allowing the liquid cultures to grow through one or two cell generations (about 6 days) on an amino acid mixture (M-2)[8] lacking arginine. Upon subsequent transfer of these cultures to a similar medium which contained arginine uniformly labelled with ^{14}C and 3·5 μmoles of carrier arginine per 100 ml, there was a rapid uptake of ^{14}C by the cells (Fig. 1). Under these conditions the growth medium was depleted of arginine in about 24 hr, and total ^{14}C uptake proceeded linearly over a 12-hr period.[9] Formation of $^{14}CO_2$ was very small, but in the presence of ^{14}C-valine or ^{14}C-leucine we did not detect any $^{14}CO_2$ at all in cells incubated similarly for 24 hr. Cultures

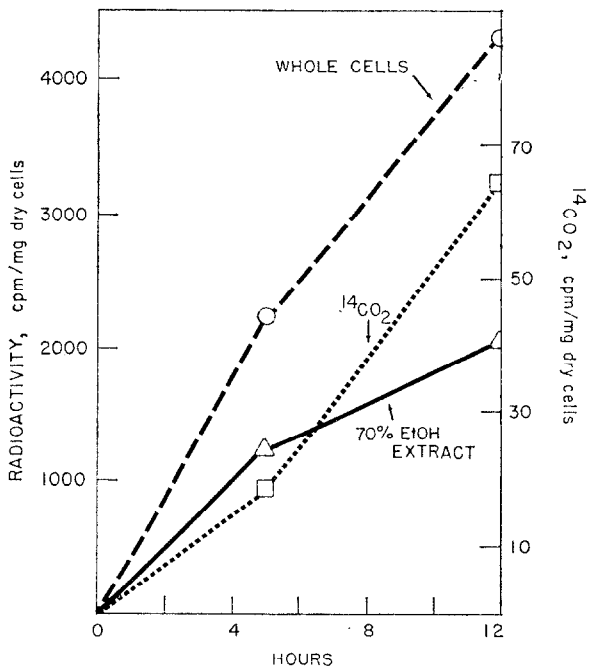

FIG. 1. UPTAKE OF ^{14}C-ARGININE (UL) BY H 50-7209 SUGARCANE CELLS AND EVOLUTION OF $^{14}CO_2$ DURING A 12-hr INCUBATION PERIOD.
Specific activity of ^{14}C-arginine (UL), 226 mc/mmole, 3 μc added per culture.

incubated with guanido-labelled, as well as those incubated with uniformly labelled arginine, showed some loss of $^{14}CO_2$, so that both decarboxylation and formation of CO_2 from the guanido group appear to be involved. The difference in radioactivity between whole cells and ethanol extracts is partly the result of protein formation. Incorporation into newly formed proteins accounted for about 8 per cent of the arginine assimilated by the cells after the first 2 hr of incubation; the protein radioactivity had increased to 15 per cent of the total count after 12 hr incubation.

Cell cultures were incubated with ^{14}C-arginine (UL) for 5 min to 120 min. On autoradiography, extracts of the dried cells chromatographed in solvent A (see Experimental) revealed three predominant radioactive products. One of these was readily identifiable as free arginine

[9] A. MARETZKI, L. G. NICKELL and M. THOM, *Physiol. Plantarum* **22**, 117 (1969).

(R_f 0·21), the second cochromatographed with guanidine (R_f 0·38), and the third has been identified as *N*-carbamylputrescine (R_f 0·66).

Identification of guanidine was based solely on chromatographic comparisons because the small quantities produced in the cells made isolation and chemical characterization impractical. An authentic sample of guanidine HCl superimposed exactly with the radioactive product from cell extracts when a two-dimensional system (solvents A and B) and a one-dimensional system, consisting of *n*-BuOH, methyl ethyl ketone, NH_3, water (5:3:1:1)[10] were employed.

Chromatographic analyses of acid hydrolysates of cell proteins showed conversion of some labelled arginine to proline and glutamic acid (< 10 per cent of the total count of the hydrolysates after incubation of the cells for 24 hr).

Isolation and identification of *N*-carbamylputrescine is described in Experimental. The spent incubation medium contained no *N*-carbamylputrescine even after cells were incubated with ^{14}C-arginine for 24 hr or longer.

The effect of exogenous arginine on the formation of *N*-carbamylputrescine in sugarcane cells was determined from the relative intra-cellular concentrations of arginine and *N*-

TABLE 1. RELATIVE CONCENTRATIONS OF ARGININE AND *N*-CARBAMYLPUTRESCINE IN THE ETHANOL-SOLUBLE EXTRACT FROM H 50-7209 SUGARCANE CELL CULTURES WHICH WERE GROWN FOR 3 weeks IN THE PRESENCE OR ABSENCE OF EXOGENOUS ARGININE

Medium (White's + 2,4-D + sucrose + M-2)	Dry cells (mg)	Concentration (μmoles/mg dry cells)	
		Arginine	*N*-Carbamylputrescine
Minus arginine	62·6	0·0077	0·0130
Plus 33 μmoles arginine per 100 ml medium	124·8	0·0123	0·0290

carbamylputrescine in cells that had been incubated for 3 weeks, either in the presence or absence of added arginine (Table 1). Within the cells the ratio of arginine to *N*-carbamylputrescine did not change, despite the fact that cell growth (measured as weight of dry cells) was 50 per cent less in the absence of added arginine than it was when arginine was included at a concentration of 33 μmoles per 100 ml of medium. The ratio of arginine to *N*-carbamylputrescine, therefore, remained unaffected by an excess supply of arginine, and it appeared to be a normal product of arginine metabolism in sugarcane cells of variety H 50-7209.

The increases in specific activity of arginine and *N*-carbamylputrescine were determined in the ethanol-soluble pool of the cane cells. Prior to introduction of ^{14}C-arginine, the cultures were grown for two cell generations on a synthetic medium lacking arginine (Fig. 2). Samples were removed from the cultures every 15 min for measurement of total ^{14}C uptake by the cells, and radioactivity in arginine and *N*-carbamylputrescine isolated from the ethanol-soluble fraction. The specific activity of arginine increased rapidly during the first 60 min at a rate which paralleled the uptake from the medium. There has been no indication in any of our experiments that arginine is converted to other products prior to its appearance in the endogenous soluble pool. We have found no evidence of extracellular enzymes capable of producing *N*-carbamylputrescine from arginine. Within the cells the conversion of arginine to

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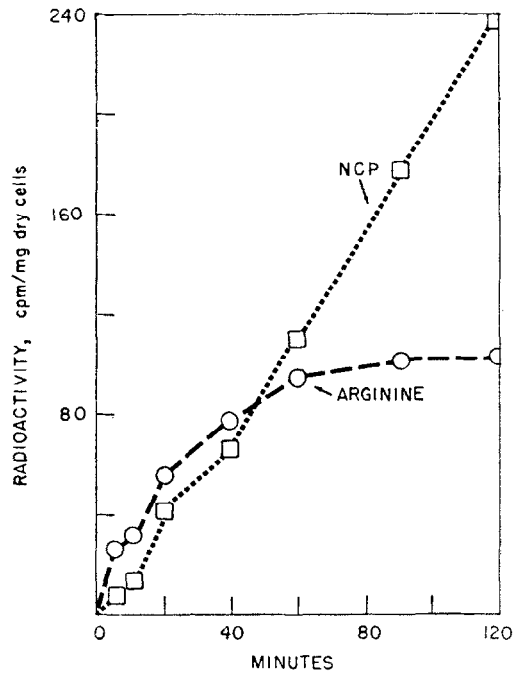

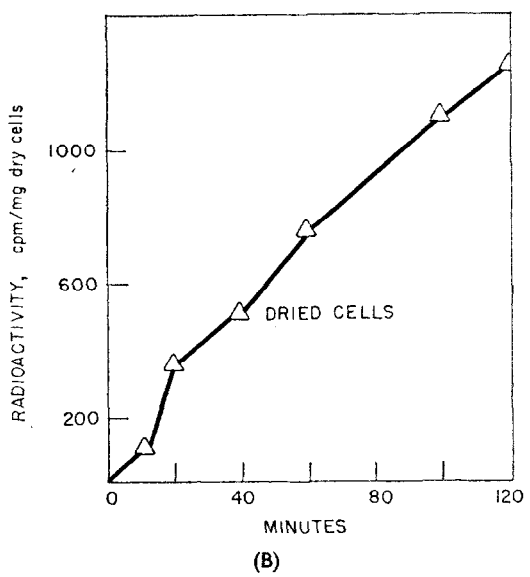

FIG. 2. A, INCREASE IN SPECIFIC ACTIVITY (IN TERMS OF DRY CELL WEIGHT) OF ARGININE AND *N*-CARBAMYLPUTRESCINE (NCP) IN SUGARCANE CELLS OF H 50-7209 INCUBATED IN A MEDIUM CONTAINING ^{14}C-ARGININE (UL) OVER A PERIOD OF 120 min. B, INCREASE IN TOTAL RADIOACTIVITY IN DRIED CELLS OVER THE SAME PERIOD.

N-carbamylputrescine proceeds readily. Radioactivity was detectable in *N*-carbamylputrescine 10 min after cells were exposed to ^{14}C-arginine. In terms of cell weight, a linear

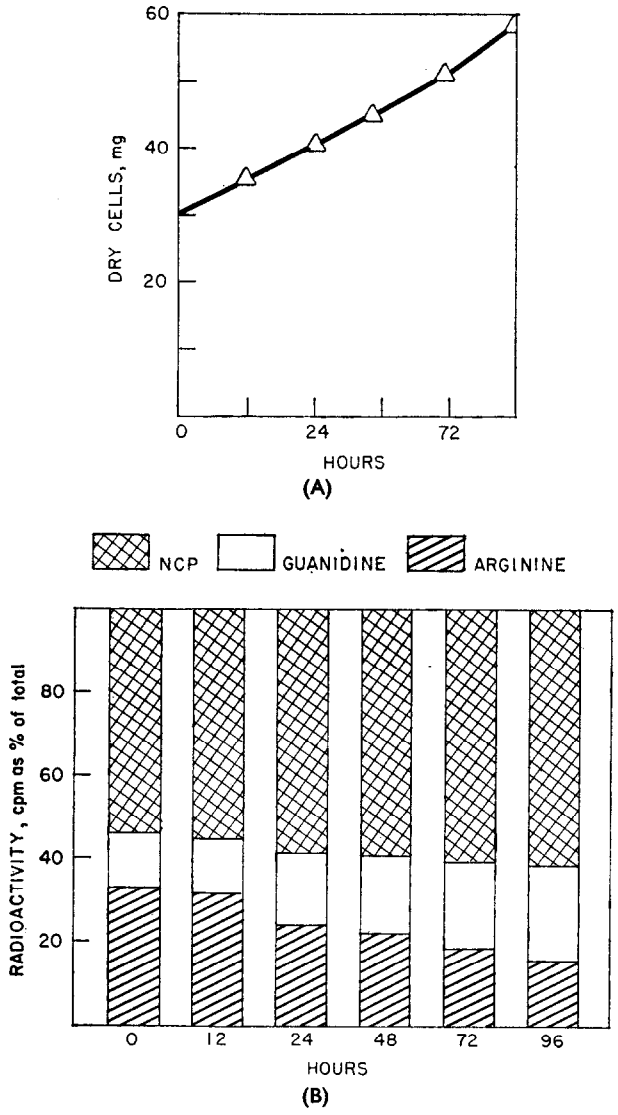

FIG. 3. SUGARCANE CELLS OF H 50-7209 INCUBATED FOR A 96-hr PERIOD FOLLOWING THEIR REMOVAL FROM A MEDIUM CONTAINING ^{14}C-ARGININE (UL) ON WHICH THESE CELLS WERE INCUBATED FOR 24-hr. A, INCREASE IN WEIGHT OF DRY CELLS PER 100 ml OF MEDIUM DURING THE POST-LABELLING PERIOD. B, CHANGE IN DISTRIBUTION OF RADIOACTIVITY OF *N*-CARBAMYLPUTRESCINE, GUANIDINE, AND ARGININE DURING THE POST-LABELLING PERIOD.

increase of *N*-carbamylputrescine radioactivity continued for at least 2 hr, whereas the activity of free arginine reached an equilibrium after the first hour of incubation.

Cell cultures were preloaded with ^{14}C-arginine for a period of 24 hr for equilibration in the presence of supplementary cold arginine before returning them to normal synthetic medium

without labelled arginine. Subsequent growth of the cultures proceeded as shown in Fig. 3a. The weight of dry cells doubled in a 96-hr period. The increase in the proportion of total cell radioactivity in guanidine and *N*-carbamylputrescine and the corresponding reduction of the proportion in arginine was followed during this time (Fig. 3). The utilization of arginine for protein synthesis would be an additional factor accounting for the decrease of free arginine. It should be emphasized that arginine derived from hydrolysed proteins could also have been utilized for the formation of guanidine and *N*-carbamylputrescine.

The incubation of sugarcane cell cultures with either guanido-labelled arginine or ureido-labelled citrulline permitted a comparison of the resulting specific activities in free arginine and *N*-carbamylputrescine (Table 2). Almost 50 per cent of the added ^{14}C-arginine was

TABLE 2. SPECIFIC ACTIVITIES OF ARGININE, CITRULLINE AND *N*-CARBAMYLPUTRESCINE RESULTING FROM INCUBATION OF H 50-7209 SUGARCANE CELLS WITH EITHER ^{14}C-ARGININE (GUANIDO-LABELLED) OR ^{14}C-CITRULLINE (UREIDO-LABELLED) FOR 24 hr IN DUPLICATE EXPERIMENTS

	Specific activity (cpm × 10^{-5}/μmole)			
	^{14}C-Arginine*		^{14}C-Citrulline†	
Chromatographic isolate from cell extracts	1	2	1	2
Arginine	4·296	4·681	0·153	0·263
Citrulline	—	—	0·701	1·475
N-carbamylputrescine	0·014	0·039	0·032	0·044
	Total radioactivity (cpm × 10^{-5}/50 ml)			
Media before incubation	20·06	23·16	32·56	29·32
Media after incubation	9·25	9·11	21·89	19·41

* Guanido ^{14}C, 13 mc/mM.
† Ureido ^{14}C, 3 mc/mM.
Arginine determined by Sakaguchi method.[11]
Citrulline determined by modified diacetyl monoxime method.[12]
N-carbamylputrescine determined by ninhydrin method.[13]

taken up by the cultures, while only about 30 per cent of the added ^{14}C-citrulline was taken up by the cells during a 24-hr incubation period, although comparable total concentrations of added arginine and citrulline were used. In spite of this difference in uptake, more *N*-carbamylputrescine was produced from citrulline than from arginine, thus suggesting the possibility that *N*-carbamylputrescine formation can proceed via citrulline instead of agmatine.

This report concerns itself primarily with the fate of arginine in sugarcane cell cultures. However, reference should also be made to the fact that cut sugarcane leaves placed in a solution containing ^{14}C-arginine for 3 hr, either in the sunlight or in darkness, converted 2–3 per cent of the total incorporated radioactivity into *N*-carbamylputrescine during this

[11] Y. IZUMI, *Anal. Biochem.* **12**, 1 (1965).
[12] D. HUNNINGHAKE and S. GRISOLIA, *Anal. Biochem.* **16**, 200 (1966).
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period. Another 5 per cent of the radioactivity was recovered as *N*-carbamylputrescine from the solution; apparently *N*-carbamylputrescine was translocated downward.

DISCUSSION

The catabolic pathways of arginine found in pine tissues,[14] fungi,[15] or bacteria[16] have not been detected in sugarcane cell cultures. The formation of free guanidine which takes place as a consequence of arginine metabolism in sugarcane cells, has so far not been reported in other higher plants. However, the presence of guanidine in higher plants has been shown.[17]

Direct cleavage of the arginine molecule to produce guanidine would necessitate postulating the formation of α-aminovaleric acid or some logical degradation product. Our chromatographic evidence with cell extract failed to demonstrate the formation of α-aminovaleric acid during incubation with labelled arginine. Guanidine synthesis could occur via cleavage of arginine by an oxidative pathway with the formation of glutamic semialdehyde as an intermediate, or by reduction of urea formed from arginine in the presence of arginase. As

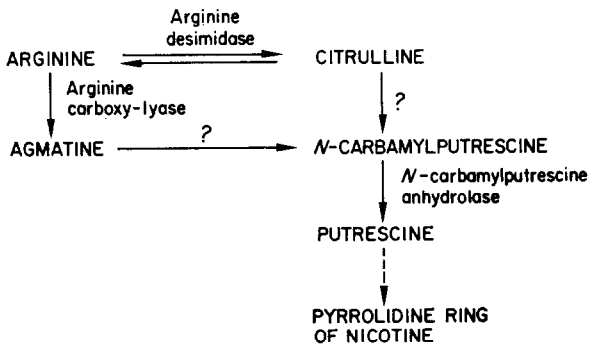

FIG. 4. METABOLIC RELATIONSHIP OF ARGININE AND PUTRESCINE.

yet none of the required intermediates, urea, ornithine or glutamic semialdehyde has been detected in the cane cell extracts. However, the formation of glutamic acid and proline from labelled arginine added to the cells was demonstrated in protein hydrolysates of the cells. These substances could be produced by either of the suggested pathways.

The presence of *N*-carbamylputrescine was unexpected since neither agmatine, the normal precursor, nor putrescine, its degradation product, was detectable in cane cell extracts. *N*-carbamylputrescine thus appears to be an end product of arginine metabolism in sugarcane cells. This is in contrast to the situation in several other plants in which it has been shown that *N*-carbamylputrescine is an intermediate in the pathway to putrescine formation.[5]

The interrelationships in Fig. 4 show that two alternative routes of *N*-carbamylputrescine synthesis from arginine are possible. In one, decarboxylation of arginine precedes desimidation, giving rise to agmatine. In the other, arginine desimidase, an enzyme recently reported in *Chlorella*,[18] may produce citrulline, which could then be decarboxylated to give

[14] R. L. BARNES, *Nature* **193**, 781 (1962).
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[18] J. SHAFER and J. F. THOMPSON, *Phytochem.* **7**, 391 (1968).

N-carbamylputrescine. We have been able to show that citrulline can serve as an effective precursor for *N*-carbamylputrescine; however, we are unable to determine from our data whether the alternative pathway also functions in sugarcane cells.

The progressive increase of *N*-carbamylputrescine concentration in the EtOH-soluble fraction, continuing for at least twice as long as the increase in arginine, indicates that this substance is metabolized very slowly. Furthermore, *N*-carbamylputrescine is not excreted by the cells into the surrounding medium. The mobility of the *N*-carbamylputrescine in the sap of fresh leaf tissue suggests that in the intact plant it might be translocated to the root system.

Neither *N*-carbamylputrescine nor guanidine appear to provide sugarcane cells with the growth stimulus which led to our investigation of arginine metabolism in this plant. The cause for the arginine effect on growth is believed to lie elsewhere and remains to be determined.

EXPERIMENTAL

Materials and Methods

Sugarcane cell cultures of variety H 50-7209 were used. The liquid cultures originated from callous tissue of parenchyma explants.[19] The cell strain had been grown continuously in liquid culture over a period of several years, and stocks, transferred at monthly intervals, had been maintained on a White's basal mixture[20] supplemented with yeast extract. Satisfactory cell growth can be obtained also on a chemically defined medium consisting of a White's basal mixture supplemented by a mixture of sixteen amino acids (M-2).[8] This synthetic medium permitted depletion of exogenously supplied arginine in the cells, and facilitated the subsequent introduction of ^{14}C-arginine into the culture medium.[9] Cells were grown in the presence of the radioisotope either with or without added carrier arginine. In the pertinent experiments, CO_2 evolved by the cell cultures was trapped in 1 N NaOH and converted to $BaCO_3$ for radioisotope counting. Radioactivity was determined on aluminum planchets with a thin-window gas flow detector in a Beckman Low-Beta II Counter.

At the end of the incubation period, cells were harvested by suction filtration, washed with water and lyophilized. Ethanol extracts (70 per cent) were chromatographed in solvent A (NH_3, *n*-PrOH; 3:7) or B (phenol saturated with water) after concentration. *N*-Carbamylputrescine was separated from other ^{14}C-labeled cell constituents in the ethanol extracts by passing the concentrated solution (adjusted to pH 7·5) through AG-50 × 8, H^+ form (Bio Rad Labs., Richmond, Calif.). Arginine was eluted with 1 N NH_4OH and the *N*-carbamylputrescine was retained quantitatively on the resin until the column was eluted with 2 N NH_4OH. The concentrated eluate was chromatographed on Whatman 3MM paper with solvent A, and the section corresponding to *N*-carbamylputrescine (R_f 0·66) was eluted, evaporated, and crystallized from aqueous ethanol. Two-dimensional chromatography in solvents A and B followed by autoradiography of the paper, attested to the radiopurity of the preparation. The product of repeated ethanol crystallizations was identified by means of mass spectrometry and i.r. and NMR spectral analyses. An authentic sample of *N*-carbamylputrescine, obtained through the kindness of Dr. T. A. Smith (Long Ashton Research Station, Bristol), was chromatographically identical to our preparation.

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