Plant Physiol. (1989) 90, 1378–1381
0032-0889/89/90/1378/04/$01.00/0

Received for publication October 7, 1988
and in revised form March 21, 1989

Polyamine Synthesis in Maize Cell Lines[1]

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ABSTRACT

Uptake of [^{14}C]putrescine, [^{14}C]arginine, and [^{14}C]ornithine was measured in five separate callus cell lines of *Zea mays*. Each precursor was rapidly taken into the intracellular pool in each culture where, on the average, 25 to 50% of the total putrescine was found in a conjugated form, detected after acid hydrolysis. Half-maximal labeling of each culture was achieved in less than 1 minute. Within this time frame of precursor incorporation, only putrescine derived from arginine was conjugated, indicating that putrescine pools derived from arginine may initially be sequestered from ornithine-derived putrescine. The decarboxylase activities were measured in each culture after addition of exogenous polyamine to the growth medium to assess differential regulation of the decarboxylases. Arginine and ornithine decarboxylase activities were augmented by added polyamine, the effect on arginine decarboxylase being eightfold greater than on ornithine decarboxylase. Levels of extractable ornithine decarboxylase were consistently 15- to 100-fold higher than arginine decarboxylase, depending on the titer of extracellular polyamine. Taken as whole the results support the idea that there are distinct populations of polyamine that are initially sequestered after the decarboxylase reactions and that give rise to separate end products and possibly have separate functions.

Polyamine synthesis in higher plants can be initiated by the decarboxylation of either arginine or ornithine (15, 18, 19). Ornithine decarboxylation gives rise to putrescine directly; arginine decarboxylation yields agmatine, which is subsequently converted to putrescine by agmatine iminohydrolase and *N*-carbamoylputrescine amidohydrolase activities (13, 17, 24). In tobacco, the two decarboxylases are developmentally controlled with ODC[2] predominating during ovary development (3, 14) and the arginine pathway predominating in leaves, stems, and roots (7, 9, 14). The two pathways are not regulated in the same fashion in cell culture; ADC is induced by a variety of environmental stresses (6) and in tobacco cultures is feedback-regulated at the level of synthesis by exogenous polyamines (7). ODC in culture is unaffected by exogenous polyamine availability and does not respond to adverse growth conditions. Stress regulation of ADC, resulting in putrescine accumulation, has also been observed in a variety of whole plants (6, 12, 16). Tobacco suspension cells are generally unaffected by inhibition of ADC but are killed by inhibition of ODC (7, 8). These results have suggested that in tobacco cells polyamines derived from ornithine are providing an essential metabolic function which cannot be substituted by polyamines derived from arginine. This further suggests that in tobacco there may be distinct populations of putrescine and other polyamines with different functions and metabolic fates.

In this paper, we have investigated the initiation of polyamine synthesis in monocot callus cell lines using precursor labeling of intracellular putrescine. This analysis was done using five different maize callus cell lines. Maize calli were used because of their relatively high rate of uptake of amino acids as well as the extensive characterization of hydroxycinnamic acid amides of the polyamines (HCAs) previously reported (10, 11, 18). We have measured precursor labeling of conjugates of the polyamines as a metabolic marker to determine if putrescine populations derived from either arginine or ornithine can be distinguished on the basis of amide synthesis. Labeling the polyamine part of the HCAs with either [^{14}C]arginine or [^{14}C]ornithine can distinguish separate populations of putrescine. This supports the idea that the two pathways for putrescine production have different metabolic functions during growth and development. The arginine pathway is the sole source of putrescine for conjugation to hydroxycinnamic acids; the ornithine pathway produces free putrescine, which is not detectibly converted to a conjugated form. In addition, the maize cell lines characterized here were devoid of feedback regulation by exogenous polyamines on the decarboxylase activities.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Callus cultures were generously provided by Dr. Jon Duvick and Dr. Steve Briggs of Pioneer Hi-Bred International, Johnston, Iowa, Briefly, *Cochliobolus carbonum*-susceptible maize (*Zea mays* L.) inbred K61 and hybrid (Pr × K61, both hm1/hm1), were crossed by B73 derived inbreds Pioneer (R) T66 and R21 (both Hm1/Hm1) to produce an F_1 having a high embryo culture response (22). F_1 plants were selfed, and immature F_2 embryos of the segregating population were cultured. Embryos excised from half ears at 12 to 14 d postpollination were cultured on embryo initiation medium in the dark (23). Friable embryogenic calli arising from calli giving a type II response after 14 d were transferred at weekly intervals to callus maintenance medium (23) for increase. Cultures were maintained at 28°C in the dark and transferred to fresh medium at 1- to 2-week intervals once stable cultures were obtained.

[1] This research was supported by U.S. Department of Agriculture grant 86-CRCR-1-2274 and National Science Foundation grant DCB-8796274.

[2] Abbreviations: ODC, ornithine decarboxylase; ADC, arginine decarboxylase; HCAs, hydroxycinnamic acid amides.

Enzyme Assays

Cells were collected by vacuum filtration, weighed, and then homogenized with a Polytron at a ratio of 1 mL homogenization buffer (40 mM sodium phosphate, 10 mM EDTA, 5 mM DTT 0.04 mM pyridoxal phosphate, pH 7.5) per fresh weight gram of cells. The homogenate was clarified by centrifugation at 10,000*g*. Two hundred microliters of a 10,000*g* supernatant was first spun-dialyzed through Sepharose CL-6B (Sigma) at a ratio of 200 μL homogenate per 0.5 mL of packed Sepharose beads equilibrated in homogenization buffer. Centrifugation was for 2 min at 2,000*g* and was performed immediately before the assay. The eluate was added to 10 μL of labeled substrate; the cap of the reaction tube (5 mL scintillation vial) contained a paper filter (Whatman 3MM) to which 30 μL of methylbenzythonium hydroxide (Sigma) was applied as a CO_2 trap (7). Reactions were allowed to proceed at 37°C for 15 min (V_{max} conditions), whereupon the reaction was stopped by the addition of 100 μL of 1 M KH_2PO_4. The tubes were resealed and allowed to incubate at room temperature for 2 h. Each filter was then counted in Aquasol 2 (New England Nuclear). Nonenzymic decarboxylation was consistently 5 to 10% of experimental values. [1-^{14}C]Ornithine (58.9 mCi/mMol) and *S*-adenosyl[1-^{14}C]methionine (57.6 mCi/mMol) were from New England Nuclear; [U-^{14}C]arginine (336 mCi/mmol) was from Amersham.

Labeling, Extraction, and Analysis of Polyamines

Friable callus pieces that had been subcultured for 1 week on fresh plates (about 0.1 g dry weight) were picked and immersed in 1 mL liquid medium. After gentle vortexing to disperse the cells, an aliquot was taken to determine the dry weight, radiolabel was added to a final concentration of 0.2 μM and the cells were allowed to incubate at room temperature for varying times. Seventy nCi of [U-^{14}C]arginine were added, 55 nCi of [1,4-^{14}C]putrescine for each time point. Cells were diluted in 10 mL ice cold water, harvested by vacuum filtration, and homogenized using a Polytron in 2 mL cold 10% perchloric acid. After 30 min on ice, the extracts were centrifuged for 10 min at 30,000*g*. Three aliquots (200 μL each) of the supernatant were sealed in glass ampoules with 200 μL 12 N HCl. After 18 h at 110°C, the hydrolysates were dried under a stream of air at 85°C then resuspended in 200 μL 10% perchloric acid. Replicates of the nonhydrolyzed supernatant (soluble, free polyamines) and the hydrolyzed supernatant (soluble, conjugated polyamines) were dansylated and chromatographed according to the procedure of Flores and Galston (5). Dry weight was determined after baking suspension cells in a vacuum oven at 80°C overnight. Dansyl polyamines were visualized by UV fluorescence and quantified by fluorimetry (Perkin-Elmer model LS3 spectrofluorimeter at 350 nm excitation, 495 nm emission) after scraping the polyamine bands into 2 mL of ethyl acetate. Radiolabeled dansyl polyamines were counted after dilution of the ethyl acetate extract into 6 ml Aquasol II using a Beckman scintillation counter. Radiolabel in polyamines was derived from either [1,4-^{14}C]putrescine (104.6 mCi/mmol), [U-^{14}C]ornithine (291.2 mCi/mmol) or [U-^{14}C]arginine (348 mCi/mmol), all from New England Nuclear.

RESULTS

Uptake and Use of Polyamine Precursors

Uptake of [^{14}C]putrescine was measured in each of the cell lines as was conjugation of labeled free putrescine *in vivo*. In general, all cultures converted 20 to 30% of the labeled intracellular free putrescine into the conjugated form after 8 min (Fig. 1). Each culture contained between 4 and 7 nmol free putrescine and 5 to 10 nmol soluble conjugated putrescine per mg dry weight. The resulting specific activity of the intracellular putrescine was 20,000 to 40,000 cpm/μmol after 8 min of labeling. Virtually all of the extracellular putrescine was taken up by the cells in 8 min. No labeled putrescine was detected in the growth medium or in the perchloric acid insoluble hydrolysate at the 8 min time point (see "Materials and Methods"). Maximum labeling of the free and conjugated putrescine pools occurred after 500 s and 240 s, respectively; however, half-maximal labeling for each population occurred in less than 30 s, suggesting that uptake of putrescine in these cell lines occurred very rapidly.

Uptake and decarboxylation of both [U-^{14}C]arginine and [U-^{14}C]ornithine was measured and proved to be similar in each culture (Fig. 2). Arginine uptake was consistantly 8 to 10 times that of ornithine. Typical values were 200,000 cpm/100 mg dry weight in intracellular arginine and 30,000 cpm/100 mg in ornithine (data not shown). The much greater total uptake of arginine presumably reflects its use in protein biosynthesis. Conversion into free putrescine of the [^{14}C] arginine pool at any time point accounted for approximately 1% of the total intracellular label. Conversion into putrescine of intracellular [^{14}C]ornithine was approximately 7.5% of the total. Consequently, since each culture contained similar

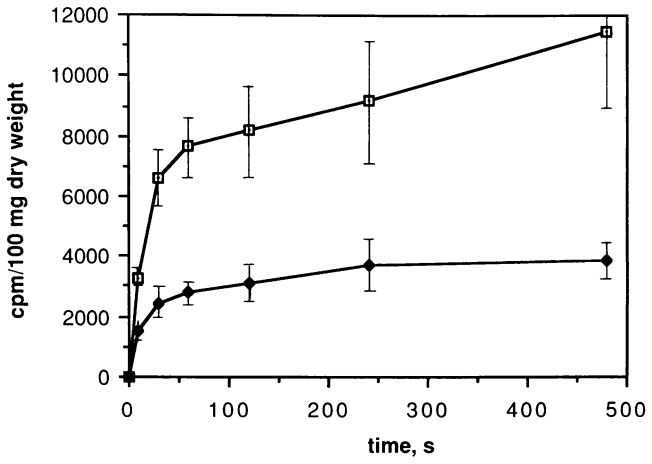

Figure 1. Uptake and conjugation of [^{14}C]-putrescine in maize cell lines. Pieces of callus were dispersed and labeled with 0.2 μM [^{14}C] putrescine for various times as described in "Materials and Methods." Putrescine was measured by dansylation and TLC before and after acid hydrolysis yielding free and free + conjugated fractions. The results are displayed as the mean ± SD of the values derived from five different cultures. □, free intracellular putrescine; ♦, conjugated putrescine.

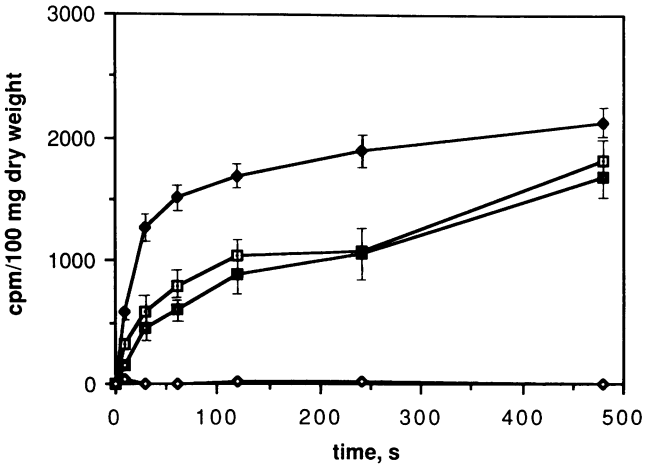

Figure 2. Uptake and conjugation of [^{14}C]ornithine and [^{14}C]arginine in maize cell lines. Calli were labeled with 0.2 μM [^{14}C]ornithine or [^{14}C]arginine for various times. Putrescine was measured by dansylation and TLC before and after acid hydrolysis yielding the free and free + conjugated fractions. The results are displayed as the mean ± SD of the values derived from five different cultures. □, free putrescine derived from arginine; ♦, free putrescine derived from ornithine; ■, conjugated putrescine derived from arginine; ◇, conjugated putrescine derived from ornithine.

amounts of putrescine per mg dry weight, specific labeling of free putrescine by either [^{14}C]arginine or [^{14}C]ornithine were nearly equal. Conversion of putrescine labeled by arginine or putrescine labeled by ornithine was also measured by dansylating extracts after acid hydrolysis. Within the time frame of the experiments (*e.g.* 8 min of labeling), only [^{14}C]arginine contributed to the label in conjugates (Fig. 2). Approximately 50% of the labeled putrescine derived from arginine was contained in the acid hydrolysate. Labeled putrescine derived from ornithine was not present in the acid hydrolysate fraction.

Effects of Exogenous Polyamines on Decarboxylase Activities

The decarboxylase activities were measured in cultures that had been incubated in plain liquid medium or medium containing 1 mM putrescine, spermidine, or spermine for 6 h. The results (Table I) show that in the absence of added polyamine, ODC activities were 100-fold higher than ADC and *S*-adenosylmethionine decarboxylase. Incubation in putrescine appears to have either stabilized ODC and ADC, stimulated their activity, or induced their synthesis. A similar effect was observed with exogenous spermidine although the magnitude was significantly less than the putrescine effect. Adding polyamine after homogenization had no effect on the activities. There was no feedback inhibition by exogenous polyamines on any of the decarboxylases. ADC and ODC were both inhibited *in vitro* by their difluoromethyl derivatized substrates (data not shown).

Table I. *Effect of Exogenous Polyamines on Decarboxylase Activities*

Callus pieces were dispersed in liquid medium containing either 1 mM putrescine, spermidine, spermine, or control for 6 h. After homogenization, polyamine was added to the control cultures and the supernatant was assayed for ADC, ODC, and *S*-adenosylmethionine decarboxylase activities as described in "Materials and Methods." Values presented are averaged from all cell lines based on duplicate measurements of each line.

Medium	Specific Activities			ODC:ADC
	ADC	ODC	SAMdc[a]	
	pmol CO_2/mg protein/h			
Control	5	514	5	103
Putrescine (1 mM)	73	944	7	13
Spermidine (1 mM)	10	608	6	61
Spermine (1 mM)	8	539	5	67

[a] *S*-Adenosylmethionine decarboxylase.

DISCUSSION

Since initiation of polyamine production in plants is accomplished by two decarboxylases, the focus of much recent work has been to determine their relative contribution under different growth conditions, their metabolic and developmental regulation, and the extent to which one decarboxylase can compensate for the loss, by inhibition, of the other (5, 7, 20). The arginine pathway has long been known to be augmented by many adverse growth conditions including potassium starvation, osmotic shock, and low pH (4, 6, 26, 27). Furthermore, during low pH shock in culture, inhibition of the arginine pathway compromises the ability of tobacco cells to recover, suggesting that arginine decarboxylation and perhaps putrescine accumulation are beneficial stress responses (8). Growth under stress does not appear to affect the ornithine pathway. A regulatory distinction between the two decarboxylases is found in the differential sensitivity to the availability of polyamines in the growth medium. In tobacco cultures, addition of exogenous polyamines results in loss of only ADC due to inhibition of synthesis of the enzyme. ODC is unaffected by excess polyamines (7). These results have suggested that the two pathways may be producing distinct populations of putrescine that have separate metabolic functions. In the present paper, we have used maize callus cultures to investigate these possibilities. The results demonstrate that putrescine pools can be identified on the basis of their differential conjugation. Only putrescine that is derived from arginine was used for conjugation in these maize callus cells. Within the time frame of the experiments, no ^{14}C derived from ornithine was detected in the the acid hydrolysate fraction. The short labeling times were chosen due to the possibility of converting ornithine into arginine in the urea cycle. We have not determine whether longer labeling times will significantly interconvert the label. In addition, we have made no attempt to determine the molecular structure of the conjugates in these cell lines. Previous studies (10, 11) have identified a variety of phenylpropanoids (*e.g.* cinnamic, caffeic, and ferulic acids) conjugated to polyamines in maize to form HCAs.

Previous precursor incorporation work in tobacco cell lines (1, 2, 18) has shown that both arginine and ornithine can be precursors to caffeoyl- and feruloyl-putrescine. These results are in contrast to the data presented here in which only arginine is incorporated into a conjugated fraction of total

putrescine. The difference in results may be due to the vastly different labeling times used in these studies; incorporation into tobacco HCAs was observed after 6 h of labeling (2). The experiments presented here were aimed at determining the initial rate of incorporation prior to recycling of the label or mixing of putrescine pools.

The discrepancy between the level of ADC compared to ODC (100-fold lower) and the equivalent *in vivo* conversion of arginine and ornithine into putrescine is perplexing. The result suggests that ADC may be inactivated during homogenization and extraction. In contrast to tobacco cell lines (7), however, ADC activity is not diminished by the availability of exogenous polyamine. The increased extractable ADC activity after cultures were incubated in polyamine could be due to stabilization of the enzyme (rendering it less susceptible to proteolysis during extraction, for example) or de novo synthesis. A similar, albeit less dramatic effect was observed with ODC activity. Increased recovery of ADC and ODC activities using PMSF have been reported in mung bean (1).

One possible conclusion from these results is that the two putrescine populations are derived from sequestered enzymes. Subcellular compartmentation of ADC and/or ODC could result in production of two pools which could further be linked to separate end products and metabolic pathways. We have not yet been able to separate the two decarboxylases using conventional subcellular fractionation techniques but this kind of result would be essential for establishing functional compartmentation of polyamines. Others (21, 25) have reported similar results suggesting that only arginine is a precursor to soluble conjugated polyamines as well as to the pyrrolidine alkaloids.

LITERATURE CITED

1. **Altman A, Friedman R, Levin N** (1983) Alternative metabolic pathways for polyamine biosynthesis in plant development. *In* U Bachrach, A Kaye, R Chayen, eds, Advances in Polyamine Research, Vol 4. Raven Press, New York, pp 395–408
2. **Berlin J** (1981) Formation of putrescine and cinnamoyl putrescine in tobacco cell cultures. Phytochemistry **20:** 53–55
3. **Cohen E, Malis-Arad S, Heimer YM, Mizrahi Y** (1982) Participation of ornithine decarboxylase in early stages of tomato fruit development. Plant Physiol **70:** 540–543
4. **Flores HE, Galston AW** (1982) Polyamines and plant stress: activation of putrescine biosynthesis by osmotic shock. Science **217:** 1259–1261
5. **Flores HE, Galston AW** (1984) Osmotic stress-induced polyamine accumulation in cereal leaves. I. Physiological parameters of the response. Plant Physiol **75:** 102–109
6. **Flores HE, Young ND, Galston AW** (1985) Polyamine metabolism and plant stress. *In* JL Key, T Kosuge, eds, Cellular and Molecular Biology of Plant Stress. Alan R Liss, New York, pp 93–114
7. **Hiatt AC, McIndoo J, Malmberg RL** (1986) Regulation of polyamine biosynthesis in tobacco. J Biol Chem **261:** 1293–1298
8. **Hiatt AC, Malmberg RL** (1988) Utilization of putrescine in tobacco cell lines resistant to inhibitors of polyamine synthesis. Plant Physiol **86:** 441–446
9. **Malmberg RL, McIndoo J, Hiatt AC, Lowe BA** (1985) Genetics of polyamine synthesis in tobacco: developmental switches in the flower. Cold Spring Harbor Symp Quant Biol **50:** 475–482
10. **Martin-Tanguy J, Deshayes A, Perdrizet E, Martin C** (1979) Hydroxycinnamic acid amides in *Zea mays*: distribution and changes with cytoplasmic male sterility. FEBS Lett **108:** 176–178
11. **Martin-Tanguy J, Perdrizet E, Provost J, Martin C** (1982) Hydroxycinnamic acid amides in fertile and cytoplasmic male sterile lines of maize. Phytochemistry **21:** 1939–1945
12. **Richards FJ, Coleman RG** (1952) Occurrence of putrescine in potassium deficient barley. Nature **170:** 460
13. **Sindhu RK, Desai HV** (1979) Purification and properties of agmatine iminohydrolase from groundnut cotyledons. Phytochemistry **18:** 1937–1938
14. **Slocum RD, Galston AW** (1985) In vivo inhibition of polyamine biosynthesis and growth in tobacco ovary tissues. Plant Cell Physiol **26:** 1519–1526
15. **Slocum RD, Kaur-Sawhney R, Galston AW** (1984) The physiology and biochemistry of polyamines in plants. Arch Biochem Biophys **235:** 283–303
16. **Smith TA** (1970) Putrescine, spermidine, and spermine in higher plants. Phytochemistry **9:** 1479–1486
17. **Smith TA** (1979) Arginine decarboxylase of oat seedlings. Phytochemistry **18:** 1447–1452
18. **Smith TA, Negrel J, Bird CR** (1983) The cinnamic acid amides of the diand polyamines. *In* U Bachrach, A Kaye, R Chayen, eds, Advances in Polyamine Research, Vol 4. Raven Press, New York, pp 347–370
19. **Srivenugolpal KS, Adiga PR** (1981) Enzymic conversion of agmatine to putrescine in *Lathyrus sativus* seedlings. J Biol Chem **256:** 9532–9541
20. **Tiburcio AF, Ingersoll R, Galston AW** (1985) Modified alkaloid pattern in developing tobacco callus. Plant Sci **38:** 207–212
21. **Tiburcio AF, Kaur-Sawhney R, Ingersoll RB, Galston AW** (1985) Correlation between polyamines and pyrrolidine alkaloids in developing tobacco callus. Plant Physiol **78:** 323–326
22. **Tomes D** (1985) Opportunities and limitations of the genotypic influences on establishment and plant regeneration from callus and cell cultures of crop species. *In* H Day, M Zilton, J Hollaender, eds, Biotechnology in Plant Science. Academic Press, New York, pp 3–14
23. **Tomes D, Smith OS** (1985) Effect of parental genotype on initiation of embryogenic callus from elite maize (*Zea mays* L.) germplasm. Theor Appl Genet **70:** 505–509
24. **Yanagisawa H, Suzuki Y** (1982) Purification and properties of N-carbamyl-putrescine amidohydrolase from maize shoots. Phytochemistry **21:** 2201–2203
25. **Young ND, Galston AW** (1983) Are polyamines transported in etiolated peas? Plant Phyiol **73:** 912–914
26. **Young ND, Galston AW** (1983) Putrescine and acid stress. Plant Physiol **71:** 767–771
27. **Young ND, Galston AW** (1984) Physiological control of arginine decarboxylase activity in K-deficient oat shoots. Plant Physiol **76:** 331–335