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BIOSYNTHESIS OF PYRIMIDINE NUCLEOTIDES IN CULTURED ROOT CALLUS OF *CUCURBITA PEPO*

FEN-FEN LIN AND CAROL J. LOVATT

Department of Botany and Plant Sciences, University of California, Riverside, California 92521

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SUMMARY

Callus cultures derived from roots of summer squash (*Cucurbita pepo* L. c.v. Early Prolific Straightneck) grown in the dark at 27° C on Murashige and Skoog medium supplemented per liter with 30 g sucrose, 100 mg myo-inositol, 10 mg indole-butyric acid, 2 mg glycine, 1 mg thiamin, 0.5 mg nicotinic acid, 0.5 mg pyridoxine, and 2 g Gelrite were capable of synthesizing pyrimidine nucleotides both de novo and through salvage of existing pyrimidine nucleotides and bases. Evidence that the de novo biosynthesis of pyrimidine nucleotides proceeded via the orotate pathway in this tissue included: (a) demonstration of the incorporation of $NaH^{14}CO_3$ and [$^{14}C_6$]orotic acid into uridine nucleotides (ΣUMP), and (b) demonstration that the addition of 6-azauridine blocked the incorporation of these two precursors into ΣUMP.

The synthesis of pyrimidine nucleotides through the salvage of existing pyrimidine bases and ribosides was demonstrated by measuring the incorporation of [$^{14}C_2$]uracil and [$^{14}C_2$]uridine into ΣUMP. Salvage of both [$^{14}C_2$]uracil and [$^{14}C_2$]uridine was sensitive to inhibition by 6-azauridine or one of its metabolites.

The orotic acid pathway for the de novo biosynthesis of pyrimidine nucleotides was demonstrated to be sensitive to end-product inhibition. Uridine, or one of its metabolites, inhibited the incorporation of $NaH^{14}CO_3$, but not [$^{14}C_6$]orotic acid, into ΣUMP. Evidence is presented suggesting that Aspartate carbomoyltransferase is the site of feedback control.

Key words: Cucurbita pepo; root callus; pyrimidine nucleotides; orotate pathway; end-product inhibition; pyrimidine salvage.

INTRODUCTION

The use of plant cells in culture to generate cell lines resistant to specific metabolic inhibitors has proven useful for studying metabolic pathways and their regulation, gene structure and function, intracellular compartmentalization, and phytohoromone action. The most extensively studied animal somatic cell variants are those resistant to inhibitors of pyrimidine and purine metabolism (7). It has been suggested that similar plant somatic cell variants should contribute significantly to the study of somatic cell genetics and metabolic regulation in plants (7).

Cucurbita pepo might prove to be an excellent system for studying gene expression and its regulation with regard to pyrimidine metabolism. Pyrimidine synthesis and its regulation have been intensively studied in intact cells and cell-free extracts of roots (8–11) and leaves (Rabe and Lovatt, unpublished) of *C. pepo* c.v. Early Prolific Straightneck. In addition, plants have been regenerated from callus cultures of two different tissues (pericarp wall and hypocotyl) from two different cultivars of *C. pepo* (6,19).

The efficacy of this system is dependent on the degree to which the metabolism of plant cells in culture mimic the intricacies of a tissue or an organ of the whole plant. As a prerequisite to developing cell lines of *Cucurbita pepo* resistant to pyrimidine base analogs or to inhibitors of the orotate pathway, we compared the capacity of callus derived from roots of *C. pepo* to synthesize pyrimidine nucleotides, either de novo or through the reutilization of existing pyrimidine bases or ribosides, and the regulation of the orotate pathway to that of intact roots.

MATERIALS AND METHODS

Chemicals. Radioisotopes were purchased from ICN Pharmaceuticals, Inc., Plainview, NY with the exception of [^{14}C]carbamoylphosphate, which was purchased from New England Nuclear Corporation, Boston, MA. Liguiscint (liquid scintillation cocktail) was purchased from National Diagnostics, Somerville, NJ. Murashige and Skoog salts were purchased from KC Biological, Inc., Lenexa, KA. Gelrite from Kelco Division of Merch and Co., Inc., Newark, NJ. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Callus. Seed coats of summer squash seeds (*Cucurbita pepo* L. c.v. Early Prolific Straightneck), courtesy of the Joseph Harris Co., Inc., Rochester, NY were removed and the embryos were soaked under vacuum for 10 min in 10% Purex

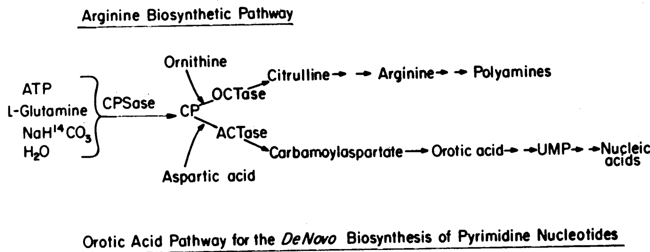

FIG. 1. Pathways for the de novo biosynthesis of pyrimidine nucleotides and arginine. *CPSase*, carbamoyl-phosphate synthase; *CP*, carbamoyl-phosphate; *OCTase*, L-ornithine carbamoyltransferase; *ACTase*, L-aspartate carbamoyltransferase.

containing two drops Tween 20/100 ml solution. Embryos were rinsed three times with sterile distilled water (dH_2O) and transferred to sterile Reinert and White (16) medium. The embryos were incubated at 27° C under 16 h light at 1000 lux (Gro-lux). After 8 d, the apical 1 cm of root was excised for initiation of callus on Murshige and Skoog (13) medium supplement per liter with 30 g sucrose, 100 mg myo-inositol, 10 mg indole-butyric acid, 2 mg glycine, 1 mg thiamin-HCl, 0.5 mg pyridoxine-HCl, 0.5 mg nicotinic acid, and 2 g Gelrite. Callus was initiated in the dark at 27° C.

Biosynthesis of pyrimidine nucleotides in intact cells. The occurrence of the complete orotic acid pathway for the de novo biosynthesis of pyrimidine nucleotides in the intact cells of root callus (500 mg fresh wt) was detected by measuring the incorporation of $NaH^{14}CO_3$ (10 m*M*, 150 μCi) or [$^{14}C_6$]orotic acid (1 m*M*, 1.5 μCi) into total uridine nucleotides converted to (UMP) by acid hydrolysis (ΣUMP) according to the method of Lovatt et al. (10). The capacity of intact cells of root callus (500 mg fresh wt) to salvage pyrimidine nucleotides and bases was assessed by measuring the incorporation of [$^{14}C_2$]uridine (1 m*M*, 1.5 μCi) or [$^{14}C_2$] uracil (1 m*M*, 1.5 μCi) into ΣUMP (10).

Identification of the site of end-product inhibition of the orotate pathway. Regulation of carbamoyl-phosphate synthase (CPSase, E.C. 6.3.55) by end products of the de novo pyrimidine pathway was investigated by measuring the influence of exogenously supplied uridine on the incorporation of $NaH^{14}CO_3$ into both ΣUMP and arginine in the intact cells of root callus according to the method of Lovatt and Cheng (9). Regulation of aspartate carbamoyltransferase (ACTase, E.C. 2.1.3.2) and dihydroorotase (DHOase, E.C. 3.5.2.3) by pyrimidine nucleotides was investigated in assays employing cell-free extracts prepared from root callus cultures according to the methods of Lovatt et al. (10) and Bresnick and Blatchford (2), respectively.

Protein content. The protein content of the supernatant used as the source of enzyme in each assay was determined according to Bradford (1) using bovine serum albumin as the standard. The buffer used in each enzyme assay served as the reagent blank.

Glucose content. Pieces of root callus were recovered by filtering the incubation mixture, washed with dH_2O, and extracted by the method of Richmond et al. (17). The glucose content of the extract was determined by a modification of the glucose-oxidase-peroxidase-*O*-dianisidine method (4,12).

RESULTS

Demonstration of the de novo biosynthesis of pyrimidine nucleotides. The occurrence of the complete orotic acid pathway (Fig. 1) for the de novo biosynthesis of pyrimidine nucleotides in the intact cells of root-callus was indicated by demonstration of the incorporation of $NaH^{14}CO_3$ and [$^{14}C_6$] orotic acid into ΣUMP (Table 1). 6-Azauridine is an inhibitor of de novo pyrimidine biosynthesis and the phosphorylated form is known to prevent the conversion of orotidine monophosphate (OMP) to UMP (3,18); 6-azauridine blocked the incorporation of both $NaH^{14}CO_3$ and [$^{14}C_6$]orotic acid into ΣUMP (Table 1).

Maximum incorporation of $NaH^{14}CO_3$ into ΣUMP per gram tissue during the 3-h incubation period was achieved with callus 3 wk after it was subcultured (Fig. 2). A significant decline in activity occurs from wk 3 to 4 ($P < 0.001$). Because of this decrease in activity in callus tissue older than 3 wk, and because the shorter subculturing period was more desirable, assays of callus routinely employed tissue more than 2 wk but less than 3 wk after subculturing. Callus of this age gave consistent results as can be seen by low degree of variation in the results of two separate experiments presented in Table 1. The average nonomoles of $NaH^{14}CO_3$ incorporated into ΣUMP per gram fresh weight tissue during the 3-h incubation period was 2.3 ± 0.3 ($\bar{x} \pm$ SE, $n = 3$).

The capacity of this tissue to provide uridine nucleotides through the activity of salvage pathways was

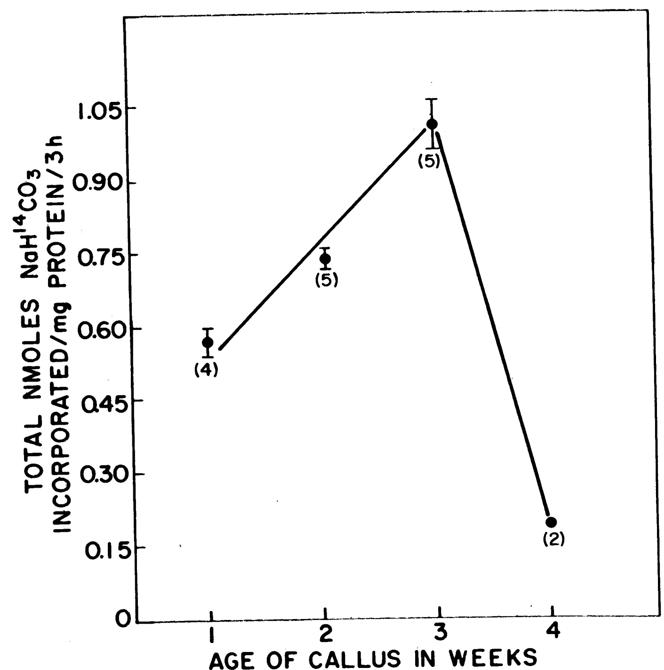

FIG. 2. Incorporation of $NaH^{14}CO_3$ into ΣUMP per gram root callus tissue during the 3-h incubation period as a function of the age of the callus in weeks after subculturing.

TABLE 1

PYRIMIDINE BIOSYNTHESIS IN INTACT CELLS OF CULTURED ROOT CALLUS

PRECURSOR	Nonomole Precursor Incorporated into ΣUMP/Gram Fresh wt, 3 h		Percent Inhibition by 6-Azauridine	
			Final Concentration	
	Experiment 1	Experiment 2	0.5 m*M*	2.5 m*M*
	de novo			
$NaH^{14}CO_3$	2.2	1.9	40[a]	—
[$^{14}C_6$]Orotic acid	6.4	4.5	—	90[a]
	Salvage			
[$^{14}C_2$]Uracil	7.9	8.9	35	40
[$^{14}C_2$]Uridine	27	45	40	40

[a]Data are the average values obtained in two to three separate experiments; all values were within 15% of the mean.

assessed by measuring the incorporation of both [$^{14}C_2$]uracil and [$^{14}C_2$]uridine into ΣUMP (Table 1), and by demonstration that this incorporation was inhibited by exogenously supplied 6-azauridine, a uridine analog and known competitive inhibitor of uridine salvage (7) (Table 1).

Regulation of the orotate pathway by end-product inhibition and identification of the regulatory enzyme. Uridine (0.5 m*M* final concentration) was added to the incubation mixture of assays measuring the incorporation of precursors into uridine nucleotides in intact callus cells. Uridine, or one of its metabolites, blocked the incorporation of $NaH^{14}CO_3$, but not [$^{14}C_6$]orotic acid into ΣUMP (Table 2). To investigate the possibility that CPSase was the site of feedback control, we measured the incorporation of $NaH^{14}CO_3$ in the presence of uridine into arginine (Arg) in intact cells of root callus (Fig. 1). Uridine did not inhibit the incorporation of $NaH^{14}CO_3$ into arginine (Table 2). Exogenously supplied ornithine dramatically increased the incorporation of $NaH^{14}CO_3$ into both ΣUMP and arginine, suggesting that ornithine stimulates the activity of CPSase in F2C. pepo callus cultures. In the presence of added ornithine, uridine inhibited the incorporation of $NaH^{14}CO_3$ into ΣUMP by more than 90%, but was without effect on the incorporation of $NaH^{14}CO_3$ into arginine (Table 2).

End-product inhibition of ACTase was demonstrated in assays employing cell-free extracts prepared from *C. pepo* root callus. UMP or UDP inhibited ACTase activity 90%, and were more effective than other pyrimidine nucleotides which ranked, when each was provided at a final concentration of 1 m*M*, as follows: UMP=UDP> UTP>CMP>CDP (Table 3). Assays of dihydroorotase activity in cell-free extracts of root callus demonstrated that this enzyme was not sensitive to inhibition by UMP (Table 3).

TABLE 2

END-PRODUCT INHIBITION OF THE OROTATE PATHWAY IN INTACT CELLS OF CULTURED ROOT CALLUS[a]

	Nanomole Precursor Incorporated into ΣUMP/Gram Fresh wt, 3 h		Nanomole Precursor Incorporated into Arg/Gram Fresh wt, 3 h
	$NaH^{14}CO_3$	[$^{14}C_6$]Orotic Acid	$NaH^{14}CO_3$
None—control	2.1	6.4	2.4
Uridine, 0.5 m*M*	0.9	5.6	1.8
Uridine, 2.5 m*M*	—	6.2	3.0
Ornithine, 5 m*M*	45.2	—	16.8
Ornithine, 5 m*M* and uridine, 0.5 m*M*	3.4	—	16.6

[a]Data are the average values obtained in three separate experiments; all values were within 15% of the mean.

Discussion

Apical 3-cm root segments excised from 2-d-old, dark germinated, summer squash seedlings (*Cucurbita pepo* L. c.v. Early Prolific Straightneck) synthesize pyrrimidine nucleotides de novo via the orotate pathway (10). Callus derived from apical 1-cm root segments of *C. pepo* and cultured on a minimal medium (13) retained the capacity to synthesize pyrimidine nucleotides de novo. De novo synthesis was demonstrated to be by the orotate pathway: (a) both $NaH^{14}CO_3$ and [$^{14}C_6$]orotic acid were incorporated into UMP, and (b) 6-azauridine inhibited the incorporation of both precursors. The activity of the orotate pathway in intact cells of *C. pepo* root callus approximately 2 wk after subculturing was 2.3 ± 0.3 (x ± SE, *n* = 3) nmol $NaH^{14}CO_3$ incorporated

TABLE 3

END-PRODUCT INHIBITION OF THE OROTATE PATHWAY IN CELL-FREE EXTRACTS OF CULTURED ROOT CALLUS[a]

Additions	Asparate Carbamyltransferase[c]	Diohydroorotase[d]
None—control	23 747	0.06
UMP	2379	0.06
UDP	2114	
UTP	4493	
CMP	6529	
CDP	17 704	
CTP	22 317	

[a]Protein content of the callus was 2.5 ± 0.2 mg/g tissue (x ± SD, *n* = 4).

[b]All end-products, when added, were provided at a final concentration of 1 m*M*.

[c]Data are presented as dpm ^{14}C-acid-stable counts per reaction mixture. The value for the no aspartate blank was 2563 dpm.

[d]Data are presented as the average change in optical density (OD) at 240 n*M* in the reaction mixture per min over a 5-min period which immediately followed a 5-min equilibration period. The assay is linear from 0 to 4.0 μmol dihydroorotate. Each change in 0.01 OD units is equal to 0.25 μmol dihydroorotate.

into uridine nucleotides per gram tissue during the 3-h incubation period. This is significantly less than the activity of the orotate pathway measured by the same method for the apical 3-cm root segments excised from 2-d-old, dark germinated *C. pepo* seedlings, in which the incorporation of $NaH^{14}CO_3$ into ΣUMP was 144 ± 10 (x ± SE, $n = 15$) nmol/g tissue during the 3-h incubation period ($P < 0.001$) (10). The lower rate of de novo synthesis of the root callus was not offset by greater capacity to salvage pyrimidine nucleotides. Salvage of $[^{14}C_2]$uracil and $[^{14}C_2]$uridine into ΣUMP in intact cells of callus tissue was 80 to 90% less than that observed for 2-d-old roots (10). Although there was 70% less protein in callus tissue than in apical 3-cm root segments excised from 2-d-old *C. pepo* plants, (2.5 and 8.3 mg protein/g tissue, respectively) when the data are expressed per milligram protein, de novo and salvage activities are still significantly different between these two systems ($P < 0.001$).

Lovatt (8) has recently shown that the capacity of intact cells of apical 3-cm root segments excised from 2-d-old, dark germinated squash seedlings to synthesize pyrimidine nucleotides de novo was reduced from 144 to 2 nmol $NaH^{14}CO_3$ incorporated into ΣUMP per gram tissue during the 3-h incubation period when 2-d-old seedlings were transferred to hydroponic culture in aerated nutrient solution for 15 h. The decline in activity of the orotic acid pathway paralleled a decline in glucose content of the roots from 5.6 to 0.25 mg glucose/g tissue. This process was reversed by supplying glucose (sucrose, fructose, or galactose) to the hydroponic culture or incubation medium. Glucose content of the root callus in assays measuring the incorporation of $NaH^{14}CO_3$ into ΣUMP was 0.09 mg glucose/g tissue, despite the fact the callus is cultured on a medium that is 88 m*M* sucrose. In intact squash roots, a level of 0.09 mg glucose/g tissue has a corresponding value of approximately 2 nmol $NaH^{14}CO_3$ incorporated/g tissue during the 3-h incubation, which is similar to the activity of the orotate pathway observed in the callus. The activity of the orotic acid pathway in root callus was demonstrated to increase as the glucose content of the tissue increased. Addition of glucose (10 m*M* final concentration) to the incubation mixture resulted in a sevenfold increase in the glucose content of the callus tissue, and in a sixfold increase in the incorporation of $NaH^{14}CO_3$ into ΣUMP.

Due to the very low activity of the de novo biosynthetic pathway, we were concerned about the efficacy of using callus tissue to study end-product inhibition. With roots from hydroponically cultured squash plants, end-product inhibition of the orotic acid pathway by added uridine could not be demonstrated unless glucose was supplied to the hydroponic culture or incubation medium (8,11). Despite the low activity of the orotate pathway in callus tissue, added uridine, or one of its metabolites, reduced the incorporation of $NaH^{14}CO_3$ into ΣUMP by more than 40%, thus making the study of end-product inhibition in callus tissue possible. In addition, de novo pyrimidine biosynthesis was demonstrated to be stimulated 20-fold by added ornithine, presumably through enhanced CPSase activity. Ornithine-stimulation of CPSase activity was demonstrated for cell-free extracts of roots from *Phaseolus aureus* (15) and leaves of *Pisum sativum* (14), but could not be demonstrated in cell-free extracts prepared from roots excised from 2-d-old squash plants (9). In intact cells of roots excised from 2-d-old squash plants, the activity of the orotate pathway was stimulated only 50 to 75% by added ornithine (9). Thus, callus tissue of *C. pepo* should prove to be an excellent system for studying the influence of ornithine on de novo pyrimidine nucleotide and arginine biosynthesis and for examining the coordinated regulation of these two pathways.

In plants, there is only one CPSase; this enzyme provides carbamoyl-phosphate for the de novo biosynthesis of both pyrimidine nucleotides and arginine (5,14,15) (Fig. 1). Despite the fact that exogenously supplied uridine inhibited the incorporation of $NaH^{14}CO_3$ into ΣUMP, uridine did not inhibit the incorporation of $NaH^{14}CO_3$ into arginine. Inasmuch as added uridine did not inhibit the incorporation of $[^{14}CO_6]$orotic acid into ΣUMP, these results suggested that feedback control of the orotate pathway is at an enzyme after the synthesis of carbamoyl-phosphate, but before the synthesis of orotic acid. In assays employing cell-free extracts prepared from *C. pepo* root callus, the activity of ACTase, but not DHOase, was inhibited by pyrimidine nucleotides (1 m*M* final concentration). Taken together, the results of this study provide evidence that ACTase is the site of physiological importance in the regulation of the orotate pathway for the de novo biosynthesis of pyrimidine nucleotides in root callus of *Cucurbita pepo*. Lovatt and Cheng (9) demonstrated that ACTase is the only enzyme of physiological importance in regulating the activity of the orotate pathway through end-product inhibition in roots of 2-d-old, dark germinated *C. pepo* seedlings.

Taken together, the results of this study provide evidence that pyrimidine synthesis in cultures of root callus from *Cucurbita pepo* will provide an excellent cell line for developing somatic cell variants to aid in the study of pyrimidine metabolism.

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