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Aspartate Carbamyltransferase[1]

SITE OF END-PRODUCT INHIBITION OF THE OROTATE PATHWAY IN INTACT CELLS OF *CUCURBITA PEPO*

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ABSTRACT

Lovatt *et al.* (1979 Plant Physiol 64: 562-569) have previously demonstrated that end-product inhibition functions as a mechanism regulating the activity of the orotic acid pathway in intact cells of roots excised from 2-day-old squash plants (*Cucurbita pepo* L. cv Early Prolific Straightneck). Uridine (0.5 millimolar final concentration) or one of its metabolites inhibited the incorporation of $NaH^{14}CO_3$, but not [^{14}C]carbamylaspartate or [^{14}C]orotic acid, into uridine nucleotides (ΣUMP). Thus, regulation of *de novo* pyrimidine biosynthesis was demonstrated to occur at one or both of the first two reactions of the orotic acid pathway, those catalyzed by carbamylphosphate synthetase (CPSase) and aspartate carbamyltransferase (ACTase). The results of the present study provide evidence that ACTase alone is the site of feedback control by added uridine or one of its metabolites. Evidence demonstrating regulation of the orotic acid pathway by end-product inhibition at ACTase, but not at CPSase, includes the following observations: (a) addition of uridine (0.5 millimolar final concentration) inhibited the incorporation of $NaH^{14}CO_3$ into ΣUMP by 80% but did not inhibit the incorporation of $NaH^{14}CO_3$ into arginine; (b) inhibition of the orotate pathway by added uridine was not reversed by supplying exogenous ornithine (5 millimolar final concentration), while the incorporation of $NaH^{14}CO_3$ into arginine was stimulated more than 15-fold when both uridine and ornithine were added; (c) incorporation of $NaH^{14}CO_3$ into arginine increased, with or without added ornithine, when the *de novo* pyrimidine pathway was inhibited by added uridine; and (d) in assays employing cell-free extracts prepared from 2-day-old squash roots, the activity of ACTase, but not CPSase, was inhibited by added pyrimidine nucleotides.

CPSase[2] II (dependent on L-glutamine as a N source, not requiring *N*-acetyl-L-glutamate as a cofactor, localized in the cytosol) is the only known enzyme in plants that synthesizes CP (1, 6, 8, 9). Thus, this enzyme provides CP to both the orotic acid pathway for the *de novo* biosynthesis of pyrimidine nucleotides and the arginine biosynthetic pathway (Fig. 1). There has been considerable interest in the regulatory mechanism controlling the allocation of CP to these two pathways. Measurements employing cell-free extracts of roots excised from 2.5- to 3-d-old, dark-germinated *Phaseolus aureus* seedlings or *Pisum sativum* leaf tissue have provided evidence suggesting the coordinated regulation of *de novo* pyrimidine and arginine biosynthesis at CPSase: (a) CPSase was inhibited by end products of the *de novo* pyrimidine pathway (UMP > UDP > UTP), and (b) pyrimidine nucleotide inhibition of CPSase was partially reversed by ornithine (6, 8). The activity of ACTase has also been shown to be inhibited by end products of the orotate pathway (UMP > UDP > UTP) in assays employing partially purified enzymes from a variety of plant species (5, 7, 10). On the basis of these observations, the following strategy has been proposed for the coordinated regulation of the *de novo* pyrimidine and arginine biosynthetic pathways (6). When CP availability is low, it is believed that most of the CP available to the cell would be used by the *de novo* pyrimidine pathway. When UMP accumulated to a sufficient level, the activity of the orotate pathway would be reduced through end product inhibition of both CPSase and ACTase. With the inhibition of CPSase, ornithine would be expected to accumulate to a level that would eventually reverse the pyrimidine nucleotide inhibition of CPSase, thus providing CP for arginine synthesis. ACTase activity would still be inhibited to ensure that CP was utilized by the arginine pathway. If the level of available uridine nucleotides became too low, ACTase would be released from end-product inhibition.

Lovatt *et al.* (3) have previously demonstrated that end-product inhibition functions as a mechanism for regulating the activity of the orotic acid pathway in the intact cells of roots excised from 2-d-old squash plants (*Cucurbita pepo* L.). Uridine (0.5 mM final concentration) or one of its metabolites inhibited the incorporation of $NaH^{14}CO_3$, but not [^{14}C]carbamylaspartate or [^{14}C] orotic acid, into ΣUMP. Thus, in the intact cells of squash, regulation of pyrimidine biosynthesis by feedback control was demonstrated to occur at one or both of the enzymic reactions leading to the synthesis of carbamylaspartate.

In the present study, we sought to determine if one of these two enzymes, CPSase or ACTase, is the only site regulating the activity of the orotate pathway through end-product inhibition or if both enzymes regulate *de novo* pyrimidine biosynthesis and, in addition, arginine synthesis through a strategy similar to that described above. To do this, we developed a method to assess the activity of the arginine biosynthetic pathway by measuring the incorporation of $NaH^{14}CO_3$ into both arginine and urea in intact cells, employing the same reaction mixture that we use to measure the incorporation of $NaH^{14}CO_3$ into ΣUMP (3). We then tested the influence of added uridine, ornithine, or both on the incorporation of $NaH^{14}CO_3$ into both ΣUMP and the combined pools of arginine and urea in intact roots excised from 2-d-old squash seedlings. The results of this investigation, taken together with previous results of Lovatt *et al.* (3), suggest that ACTase is the only site of physiological importance in the regulation of the orotate pathway by end-product inhibition in this tissue.

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[2] Abbreviations: CPSase, L-glutamine-dependent carbamylphosphate synthetase (EC 2.7.2.9); CP, carbamylphosphate; ACTase, L-aspartate carbamyltransferase (EC 2.1.3.2) ΣUMP, sum of the uridine nucleotides converted to UMP by acid hydrolysis; OCTase, L-ornithine carbamyltransferase.

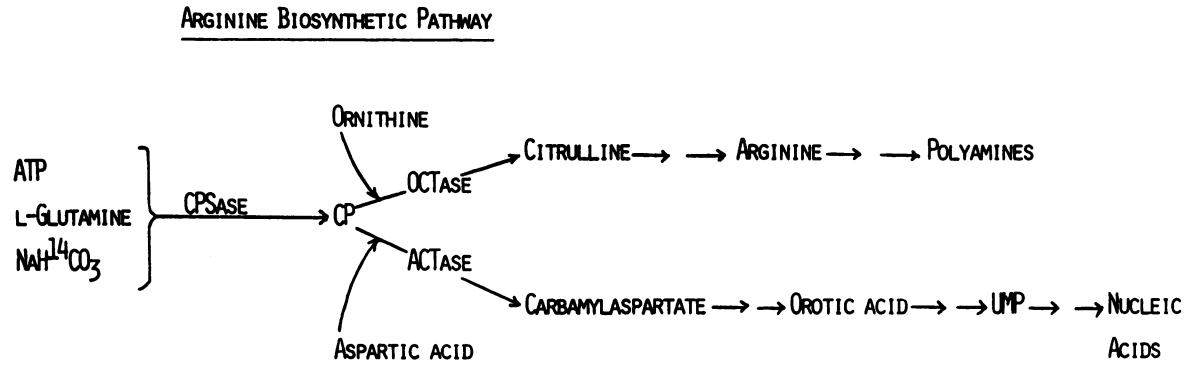

FIG. 1. Pathways for the *de novo* biosynthesis of pyrimidine nucleotides and arginine. CPSase, carbamylphosphate synthetase; CP, carbamylphosphate; OCTase, L-ornithine carbamyltransferase; ACTase, L-aspartate carbamyltransferase.

MATERIALS AND METHODS

Chemicals. All radiolabeled chemicals were purchased from ICN Pharmaceuticals, Inc. Liquiscint (liquid scintillation cocktail) was purchased from National Diagnostics. Mineral salts for Shive's nutrient solution were of analytical reagent quality. All other chemicals were purchased from Sigma Chemical Company.

Plant Material. Seeds of summer squash (*Cucurbita pepo* L. cv Early Prolific Straightneck), courtesy of the Northrup King Co., were imbibed in distilled H_2O at room temperature. After 24 h, the seeds were rinsed three times with distilled H_2O, spread evenly between two sheets of paper toweling placed in a plastic box (33 × 23 × 9.5 cm), and moistened with 7 ml of distilled H_2O. The covered box was placed in a growth chamber where the seeds germinated in the dark for 48 h at 30°C. At the end of the germination period, the roots (generally 2–4 cm long) were excised, pooled, and immediately weighed into specific aliquots which were maintained between sheets of wet paper towels until weighing was completed. Approximately seven to eight roots were needed to obtain 150 mg fresh weight tissue.

Incorporation of $NaH^{14}CO_3$ into Uridine Nucleotides and into Arginine and Urea. Samples consisting of 150 mg fresh weight of excised roots were routinely preincubated for 2 h at 30°C in 5 ml Shive's nutrient solution (5 mM $Ca[NO_3]_2$, 2 mM $MgSO_4$, 2 mM K_2SO_4, 1 mM KH_2PO_4, 1 mg Fe/L, 0.13 mg Cl/L, 1 mg Mn/L, 0.1 mg Zn/L, 0.1 mg B/L, 0.01 mg Cu/L, and 0.01 mg Na/L) adjusted to pH 7.4. At the end of the preincubation period, the nutrient solution was decanted, and the roots were immediately transferred to fresh Shive's nutrient solution supplemented with 10 mM $NaH^{14}CO_3$, 1650 dpm/nmol, and any other additives in a final volume of 5 ml, pH 7.4. Reaction mixtures were incubated for 3 h at 30°C in a water bath-shaker. Incubations were carried out in 25-ml Erlenmeyer flasks sealed with rubber stoppers fitted with a plastic center well (Kontes Glassware, Vineland, NJ) containing a filter paper wick. At the end of the incubation period, 0.5 ml of 6 N KOH was injected into the plastic center well, and the reaction was terminated by injecting 1 ml of 1.5 N $HClO_4$ into the main chamber of the flask. The $^{14}CO_2$ generated was allowed to distill from the acidified incubation mixture into the KOH in the center well for an additional 10-min incubation at 30°C. The contents of the main chamber were homogenized with a Polytron homogenizer (PCU-2, Brinkman Instruments). The Polytron probe was rinsed with 2 ml of 0.25 N $HClO_4$ which was added to the homogenate. The insoluble material was removed by centrifugation at 10,000*g* for 10 min at 0°C.

An aliquot of 1.5 ml was removed from the acid-soluble supernatant fraction for subsequent isolation of both [^{14}C]arginine and [^{14}C]urea, and the remainder of the acid-soluble fraction was boiled for 1 h at 100°C to convert the uridine nucleotides to UMP.

Isolation of [^{14}C]Uridine Nucleotides as [^{14}C]UMP. Uridine nucleotides synthesized from $NaH^{14}CO_3$ by the squash roots were isolated from the acid-soluble fraction as described previously (3).

Quantitation of the Combined Pools of [^{14}C]Arginine and [^{14}C]Urea Synthesized from $NaH^{14}CO_3$ by Squash Roots. The 1.5-ml aliquot removed from the acid-soluble supernatant fraction of the incubation mixture was neutralized to pH 7.5 with KOH and made 50 mM with $Ba(OH)_2$ in a final volume of 2.0 ml. The resulting precipitate of $BaCO_3$ was removed by centrifugation in a tabletop centrifuge (Dynac) at setting 70 for 10 min. The supernatant was made 100 mM glycine with 0.22 ml of 1 M glycine-NaOH buffer, pH 9.0, and brought to a final pH of 9.0. The final volume of the sample was recorded. A 1-ml sample was transferred to a 25-ml Erlenmeyer flask to which was added 0.2 ml of an aqueous solution containing 2 units of arginase (Sigma, from bovine liver; 1 unit catalyzes the conversion of 1.0 μmol of L-arginine to ornithine and urea per min at pH 9.5 at 37°C) and 0.2 ml of an aqueous solution containing 2 units of urease (Sigma Type III, from Jack beans; 1 μM unit liberates 1.0 μmol ammonia from urea per min at pH 7.0 at 25°C). The flasks were sealed with rubber stoppers fitted with a plastic center well containing a filter paper wick and 0.3 ml of 20% (w/v) KOH, and incubated in a water bath-shaker for 3 h at 37°C. At the end of the incubation, 1 ml of 6 N $HClO_4$ was injected into the main chamber of the flask. After an additional 30 min incubation at 37°C in the water bath-shaker, an aliquot of 0.2 ml of 0.1 M $NaHCO_3$ was injected into the main chamber of each flask. After another 30 min at 37°C, the center well and its contents were transferred to scintillation vials containing 3.7 ml H_2O. Samples were diluted with 13 ml Liquiscint, and the activity of the radioisotope in each sample was determined by using a Beckman LS 100 liquid scintillation spectrometer. Quenching was determined by an automatic external standardization system of quench analysis. A standard quench curve was determined by using a series of samples containing a known amount of ^{14}C-radioactivity and increasing amounts of chemical quencher. Results of experiments employing intact roots are expressed as nmol ± SE with the number of experiments given in parentheses.

To incubation mixtures of squash roots prepared as described

above but incubated with unlabeled $NaHCO_3$, aliquots of [^{14}C] arginine (previously purified by co-crystallization with carrier arginine, and thus, of known specific radioactivity) were added, and the reactions were immediately terminated by addition of $HClO_4$. The amount of [^{14}C]arginine recovered from each sample was determined.

Detection of Enzymic Activities. Enzymic activities were measured in cell-free extracts prepared from roots excised from 2-d-old squash plants and homogenized with a Duall all-glass conical tissue grinder (Kontes Glassware) in the specified extraction medium. All assays were based upon the measurement of incorporation of ^{14}C-labeled precursor into product.

CPSase. The activity of CPSase was assessed by two methods: (a) in the presence of excess ornithine and commercial ornithine carbamyltransferase to convert all the CP synthesized by the plant enzyme to citrulline which was isolated as described by Lovatt *et al.* (3); and (b) by trapping the CP synthesized, or the cyanate released from the breakdown of CP, as hydroxyurea by reaction with hydroxylamine as described by Levine and Kretchmer (2). The following conditions for the extraction and assay of the enzyme are based on those of Ong and Jackson (8) and O'Neal and Naylor (6). A homogenate of 1 g of roots was prepared in 5 ml of 50 mM Tris-glycine buffer (pH 8.4) containing 25 mM KCl, 25 mM $MgCl_2$, 1 mM DTT, 30% (v/v) DMSO, and 20% (v/v) glycerol. The homogenate was centrifuged at 10,000*g* at 0°C for 20 min, and the supernatant liquid was used as the source of enzyme. The activity of CPSase was measured in a 1-ml reaction mixture of the following composition: 10 mM Tris-HCl (pH 8.0); 15 mM $MgCl_2$; 10 mM L-glutamine; 10 mM ATP; 20 mM $NaH^{14}CO_3$ (1,000 or 10,000 dpm/nmol); and 0.5 ml of enzyme preparation. When citrulline was to be isolated, the lower specific radioactivity for $NaH^{14}CO_3$ was employed, and the reaction mixture also contained 10 mM L-ornithine and 2.3 units ornithine carbamyltransferase (Sigma) (1 unit catalyzes the formation of 1 μmol of citrulline/min at 37°C). After 30-min incubation at 30°C, the reaction was terminated by the addition of 3 ml of ethanol-isopropyl alcohol (1:1, v/v), and the precipitated protein was removed by centrifugation. The [^{14}C]citrulline synthesized from $NaH^{14}CO_3$ was isolated from the supernatant fluid by co-crystallization with carrier citrulline according to the procedure described by Lovatt *et al.* (3).

When the CP synthesized by the plant enzyme was to be trapped as hydroxyurea, the reaction was terminated at the end of a 30-min incubation at 31°C by the addition of hydroxylamine hydrochloride (pH 7.0) at a final concentration of 0.2 M. The vial was capped with a marble and transferred to a boiling water bath for 10 min. After cooling, 0.2 ml of 50% (w/v) TCA was added to acidify the mixture, and a small spatula, full of granulated dry ice, was added to remove residual $^{14}CO_2$. The reaction mixture was diluted to 4 ml with water, and 13 ml of Liquiscint scintillation cocktail was added. The activity of the radioisotope in each sample was determined by using a Beckman LS 100 liquid scintillation spectrometer.

ACTase. The activity of squash ACTase was assessed as described previously by Lovatt *et al.* (3). A homogenate of 1 g of excised roots in 5 ml of 0.2 M $KHCO_3$ was centrifuged at 10,000*g* at 0°C for 20 min, and the supernatant liquid was used as the source of enzyme. Each incubation mixture contained the following components at the given concentrations in a final volume of 0.5 ml: 0.1 M glycine/KOH buffer (pH 9.5); 20 mM potassium aspartate; 15 μM [^{14}C]carbamylphosphate, 0.5 μCi; and 0.2 ml of enzyme preparation. After 30-min incubation at 22°C, the reaction was terminated by the addition of 0.25 ml of 1 N HCl, and the acidified reaction mixture was baked to dryness over a boiling water bath. The residue, containing the acid-stable carbamylaspartate, was extracted with 4 ml of H_2O and diluted with 13 ml Liquiscint liquid scintillation fluor. The content of acid-stable radioactivity was determined by using a Beckman LS 100 liquid scintillation spectrometer.

RESULTS

***De Novo* Arginine-Urea Biosynthesis.** Activity of the complete pathway for the *de novo* biosynthesis of arginine was 40 ± 4 nmol ($n = 6$) $NaH^{14}CO_3$ incorporated into the combined pools of arginine and urea per g intact squash roots during the 3-h incubation period. The recovery of [^{14}C]arginine by our method was linear over a range of arginine concentrations from 10 to 1000 nmol. Average recovery was 80% over this range and, at any given arginine concentration, the standard deviation did not exceed ±7% ($n = 3$).

For comparison, the activity of the orotic acid pathway for the *de novo* biosynthesis of pyrimidine nucleotides determined from the same reaction mixture was 105 ± 10 nmol ($n = 4$) $NaH^{14}CO_3$ incorporated per g intact roots during the 3-h incubation period. For seeds of *Cucurbita pepo* L. cv Early Prolific Straightneck employed in this research, the activity of the orotic acid pathway was 30% less than that previously determined by this method for 2-d-old roots of this cultivar (3).

Influence of Added Uridine and/or Ornithine on *De Novo* Pyrimidine and Arginine Biosynthesis in Intact Roots. The addition of uridine (0.5 mM final concentration) inhibited the incorporation of $NaH^{14}CO_3$ into ΣUMP by 80% but did not inhibit the incorporation of $NaH^{14}CO_3$ into arginine (Table I). This result suggested that CPSase is not sensitive to end-product inhibition in the intact roots excised from 2-d-old *C. pepo* seedlings. In light of previous work by Lovatt *et al.* (3) indicating that regulation occurs at one or both of the enzymic reactions leading to the synthesis of carbamylaspartate in this tissue, this result further suggested that ACTase alone is the enzyme regulating the activity of the orotate pathway through end-product inhibition. Consistent with the lack of feedback control at CPSase, the inhibition of the *de novo* pyrimidine pathway by added uridine was not reversed by supplying exogenous ornithine (5 mM final concentration), while the incorporation of $NaH^{14}CO_3$ into arginine was stimulated more than 14-fold when both uridine and ornithine were present (Table I).

Finally, the incorporation of $NaH^{14}CO_3$ into arginine increased, with or without added ornithine, when the *de novo* pyrimidine pathway was inhibited by added uridine (Table I). This result was consistent with an increase in available CP that

Table I. *Incorporation of $NaH^{14}CO_3$ into Arginine and Urea and into ΣUMP by Intact Roots Incubated in the Presence of Added Uridine, Ornithine, or Both*

Assays employed 150 mg of intact roots excised from 2-d-old, dark-germinated squash seedlings. $NaH^{14}CO_3$ was provided at final concentration and specfic radioactivity of 10 mM, 1650 dpm/nmol; uridine and ornithine, when employed, were added to give final concentrations of 0.5 mM and 5 mM, respectively. The data are expressed as a per cent of the control for each experiment. Control values were 40 ± 4 nmol ($n = 6$) and 105 ± 10 nmol ($n = 4$) $NaH^{14}CO_3$ incorporated into arginine plus urea and ΣUMP, respectively, per g intact roots during the 3-h incubation.

Additions	Control			
	Arginine + Urea		ΣUMP	
	Exp 1	Exp 2	Exp 1	Exp 2
	%			
None—control	100	100	100	100
Uridine	117	115	22	17
Ornithine	1,040	1,182	178	149
Uridine, ornithine	1,613	1,400	30	42

can be accounted for only if the orotate pathway is inhibited at ACTase, but not at CPSase, when uridine is added. This reciprocal interaction between the arginine and orotate pathways is consistent with the suggested occurrence of only one CPSase in plants. Likewise, a 63% reduction in arginine pathway activity when norvaline, an ornithine analog and known inhibitor of OCTase, was supplied at a final concentration of 10 mM to assays employing intact roots resulted in a concomitant 70% increase in the incorporation if $NaH^{14}CO_3$ into ΣUMP.

Influence of Pyrimidine Nucleotides and/or Ornithine on CPSase and ACTase Activity in Cell-Free Extracts. Consistent with results employing intact squash roots, assays employing cell-free extracts prepared from roots excised from 2-d-old squash seedlings provided evidence that the activity of ACTase, but not CPSase, was regulated through feedback control by pyrimidine nucleotides. ACTase was inhibited more than 80% by the addition of UMP at a final concentration of 1 mM (Table II), while CPSase was not affected (Table III). In two separate assays, inhibition of ACTase by UMP was equal to that by UDP but more effective than other pyrimidine nucleotides which ranked, when each was provided at a final concentration of 1 mM, as follows: UMP and UDP > UTP > CMP > CDP > CTP (Table II). CPSase was not sensitive to the addition of any of these end products (Table III). UMP concentrations as low as 0.05 mM inhibited the activity of ACTase more than 70% (Table II). Surprisingly, ornithine stimulated ACTase activity, but not CPSase, and completely prevented the inhibition of ACTase by added UMP (Tables II and III).

Table II. *Aspartate Carbamyltransferase Activity*

Data are presented as dpm of ^{14}C-acid-stable counts per reaction mixure and as a per cent of the control in each experiment.

Incubation Conditions	Enzyme Source: 1:4 Dilution of the supernatant fraction of the cell-free extract[a]					
	Exp 1		Exp 2		Exp 3	
	dpm	%	*dpm*	%	*dpm*	%
Complete (control)	14,871	100	15,687	100	12,547	100
− Cell-free extract			1,468	9	1,718	14
− L-aspartate	6,688	45	5,175	33	5,245	42
+ UMP, 0.05 mM					3,568	28
+ UMP, 0.5 mM			2,834	18	3,317	26
+ UMP, 1 mM	2,362	16				
+ UDP, 1 mM	2,424	16				
+ UTP, 1 mM	3,727	25				
+ CMP, 1 mM	5,742	38				
+ CDP, 1 mM	10,573	71				
+ CTP, 1 mM	14,263	96				
+ L-ornithine, 0.1 mM					39,960	318
+ L-ornithine, 1 μM			33,664	215	34,834	278
+ UMP, 0.5 mM, and L-ornithine, 1 mM			32,233	205	34,907	278

[a] Average mg protein/g 2-d-old squash roots was 8.25 ± 0.1 (SE) (n = 7).

Table III. *Carbamylphosphate Synthetase Activity*

In experiment 1, the activity of CPSase was assessed in the presence of excess ornithine and commercial ornithine carbamyltransferase as described by Lovatt *et al.* (3). The values are given as dpm/10 mg carrier citrulline used in the isolation of [^{14}C]citrulline by co-crystallization. For experiments 2 and 3, the values are given as total nmol [^{14}C] trapped by reaction with hydroxylamine (0.2 M final concentration) per incubation mixture/mg protein during the 30-min incubation.

Incubation Conditions	Exp 1	Exp 2	Exp 3
	dpm/10 mg	*nmol product/mg protein*	
Complete (control)	484	61	53
− Cell-free extract	1	4	4
− Ornithine, − OCTase	33		
− ATP	14	20	
− L-glutamine	349	56	
+ UMP, 1 mM	554	61	49
+ UDP, 1 mM	489		
+ UTP, 1 mM	530		
+ CMP, 1 mM	598		
+ CDP, 1 mM	522		
+ CTP, 1 mM	568		
+ L-ornithine, 1 mM		53	
+ L-ornithine, 5 mM		66	48
+ L-ornithine, 10 mM		67	50
+ UMP, 1 mM, and L-ornithine, 5 mM		58	47

To test the possibility that the observed increase in ^{14}C-acid-stable counts in the ACTase assay might be due to the synthesis of [^{14}C]citrulline when ornithine was supplied exogenously, by the presence of endogenous OCTase in the supernatant fraction, we determined the level of [^{14}C]carbamylphosphate incorporated into ^{14}C-acid-stable counts in the absence of aspartate but in the presence of ornithine at concentrations from 1 to 20 mM. In each case, ^{14}C-acid-stable counts did not exceed the background level of ^{14}C-acid-stable counts observed in reaction mixtures to which no ornithine and no aspartate had been added.

Further evidence to support a direct effect of ornithine on ACTase is the observation that concentrations of ornithine greater than 10 mM inhibited the activity of the enzyme. In the presence of 20 mM ornithine, ACTase was inhibited 80%.

DISCUSSION

In assays employing intact roots excised from 2-d-old squash plants, exogenously supplied uridine blocked the incorporation of $NaH^{14}CO_3$, but not [^{14}C]carbamylaspartate or [^{14}C]orotic acid, into ΣUMP (3) but failed to block the incorporation of $NaH^{14}CO_3$ into arginine (this study). These observations taken together suggest that ACTase is a site of physiological importance in regulating the activity of the orotic acid pathway for the *de novo* biosynthesis of pyrimidine nucleotides through end-product inhibition in *C. pepo*. This possibility is supported further by the results of assays employing cell-free extracts of squash roots. ACTase, but not CPSase, was inhibited by end products of the *de novo* pyrimidine pathway. The sensitivity of squash root ACTase to the various pyrimidine nucleotide species was similar to that reported previously for mung bean root (*Phaseolus aureus*) ACTase (UMP = UDP > UTP > CMP > CDP > CTP) (7). However, unlike our observations with cell-free extracts of squash roots, UMP-inhibition of mung bean root ACTase could not be prevented by the addition of ornithine to assays employing either crude extracts or partially purified enzymes (8). In further contrast to *P. aureus*, *C. pepo* CPSase was not sensitive to feedback inhibition by added pyrimidine nucleotides nor to activation by exogenous ornithine. For both plant species, crude extracts of roots excised from 2- to 3-d-old, dark-germinated seedlings prepared with the same extraction buffer (8) were employed. In the present study, two different methods were employed to assess the activity of the squash CPSase. The procedure employing commercial OCTase required the addition of excess ornithine in the incubation mixture. Inhibition of CPSase by UMP was detected in the presence of ornithine in mung bean (8) but not in squash. However, a second method not requiring ornithine in the assay confirmed that squash CPSase was not sensitive to regulation by pyrimidine end products or ornithine.

The results of the present study, which utilized both intact cells and cell-free extracts, taken together with the results of the previous investigation by Lovatt *et al.* (3), provide strong evi-

dence that in *C. pepo* roots the only site of physiological importance in the regulation of the orotic acid pathway for the *de novo* biosynthesis of pyrimidine nucleotides by uridine or one of its metabolites is the reaction catalyzed by ACTase. We propose that feedback control of the orotate pathway at ACTase may be an alternative mechanism regulating the activity of this pathway in plants, and may be one of several strategies among plant species for modulating *de novo* pyrimidine nucleotide synthesis. The results of Ong and Jackson (8) and O'Neal and Naylor (6) obtained in experiments employing cell-free extracts of roots excised from 2.5- to 3-d-old, dark-germinated *Phaseolus aureus* seedlings or partially purified enzyme preparations of *Pisum sativum* leaf tissue, respectively, provided evidence that CPSase also serves as a site for regulation of the orotate pathway in some plant species. Further research is needed. The present investigation is only the second in which end-product inhibition of both CPSase and ACTase was examined in the same tissue of a vascular plant species, and is the first to examine the influence of pyrimidine end products on both the orotate and arginine biosynthetic pathways in intact plant cells.

The enhanced incorporation of $NaH^{14}CO_3$ into ΣUMP by the addition of ornithine to assays employing intact roots is taken as evidence that ACTase is activated by ornithine in intact roots of *C. pepo*. This interpretation is consistent with the observed stimulation of ACTase by ornithine in assays employing cell-free extracts prepared from this tissue. Exogenously-supplied ornithine also increased the incorporation of $NaH^{14}CO_3$ into arginine. Whether this enhanced activity is merely due to the provision of substrate for ornithine carbamyltransferase remains to be determined. While stimulated incorporation of $NaH^{14}CO_3$ into both ΣUMP and arginine might result if CPSase was activated by ornithine, this possibility is not supported by the results of assays employing cell-free extracts of *C. pepo*. The physiological significance of the observed activation of ACTase by ornithine, if any, remains to be demonstrated. Toward this end, it would be of value to determine: (a) the minimum size *in vivo* of the ornithine pool which will activate ACTase, and (b) the *in vivo* conditions, if any, which will result in the accumulation of ornithine to this minimum value.

Finally, the enhanced incorporation of $NaH^{14}CO_3$ into arginine when the activity of the orotic acid pathway was reduced through end-product inhibition, or into ΣUMP when the activity of the arginine pathway was inhibited by norvaline, suggests the operation of only one CPSase in squash roots. The condition of having only one CPSase and an ACTase enzyme that is sensitive to inhibition by pyrimidine nucleotides identifies the organization of the initial steps of *de novo* pyrimidine biosynthesis in *C. pepo* as similar to that of *Escherichia coli* and *Salmonella typhimurium* (4).

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