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# Regulation of Pyrimidine Biosynthesis in Intact Cells of *Cucurbita pepo*[1]

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## ABSTRACT

**The occurrence of the complete orotic acid pathway for the biosynthesis *de novo* of pyrimidine nucleotides was demonstrated in the intact cells of roots excised from summer squash (*Cucurbita pepo* L. cv. Early Prolific Straightneck). Evidence that the biosynthesis of pyrimidine nucleotides proceeds via the orotate pathway in *C. pepo* included: (*a*) demonstration of the incorporation of [$^{14}$C]$NaHCO_3$, [$^{14}$C]carbamylaspartate, and [$^{14}$C]orotic acid into uridine nucleotides; (*b*) the isolation of [$^{14}$C]orotic acid when [$^{14}$C]$NaHCO_3$ and [$^{14}$C]carbamylaspartate were used as precursors; (*c*) the observation that 6-azauridine, a known inhibitor of the pathway, blocked the incorporation of early precursors into uridine nucleotides while causing a concomitant accumulation of orotic acid; and (*d*) demonstration of the activities of the component enzymes of the orotate pathway in assays employing cell-free extracts.**

**Regulation of the activity of the orotate pathway by end product inhibition was demonstrated in the intact cells of excised roots by measuring the influence of added pyrimidine nucleosides on the incorporation of [$^{14}$C]$NaHCO_3$ into uridine nucleotides. The addition of either uridine or cytidine inhibited the incorporation of [$^{14}$C]$NaHCO_3$ into uridine nucleotides by about 80%. The observed inhibition was demonstrated to be readily reversible upon transfer of the roots to a nucleoside-free medium. Experiments employing various radiolabeled precursors indicated that one or both of the first two enzymes in the orotate pathway are the only site(s) of regulation of physiological importance.**

Most of our knowledge of pyrimidine biosynthesis has come from studies employing unicellular organisms and mammalian species. In these organisms, it has been established that pyrimidine nucleotides are synthesized *de novo* via the orotic acid pathway (Fig. 1) (for comprehensive reviews see refs. 14 and 22). Although less is known about pyrimidine metabolism in plants, available evidence indicates that the orotic acid pathway also serves as the *de novo* source of pyrimidine nucleotides in these organisms. The ability of higher plants to incorporate intermediates of the orotic acid pathway into pyrimidine nucleotides has been demonstrated previously (4, 16, 27–29, 31), and the activities of one or another of the component enzymes in the pathway have been detected in cell-free extracts of a variety of plant species (2, 13, 15, 16, 18–20, 23, 25, 26, 29, 32, 35, 37). Demonstration of the occurrence and coordination of the activities of all of the component enzymes of the orotate pathway in a single tissue source is essential to assess the physiological importance of the pathway in providing pyrimidines for plant cells and is basic to investigations into regulation of the activity of the pathway in the intact cell.

Studies of pyrimidine biosynthesis were among the first to reveal the importance of end product inhibition as a regulatory mechanism governing the activity of a metabolic pathway. The work of Yates and Pardee (36), Gerhart and Pardee (8), and Gerhart and Schachman (9) provided the early experimental data for what has become the classical model for feedback control, the inhibition of ACTase[3] from *Escherichia coli* by cytidine triphosphate. Since the initiation of those studies, there have been numerous attempts to detect such a regulatory mechanism for controlling pyrimidine biosynthesis in a variety of microbial, plant, and animal species. Feedback inhibition of virtually all of the enzymes of the orotate pathway has been reported as a result of assays employing cell-free extracts. In plants, measurements employing cell-free extracts of a variety of species have provided evidence for end product inhibition of CPSase (23, 26), ACTase (13, 20, 25, 37), and the combined activities of OPRTase and ODCase (2, 35). The operation of end product control over pyrimidine biosynthesis has not been demonstrated in the intact plant cell, a necessary step in assessing the physiological significance of the observations made with isolated enzymes or cell-free extracts. In addition, studies with intact cells might show which of these potential sites of end product inhibition is the site of primary physiological importance (6). Here, the operation of the complete orotate pathway is demonstrated in the intact cells of *Cucurbita pepo* and the occurrence of the component enzymes is verified with measurements of their catalytic activities in cell-free extracts. Regulation of the pathway by end product inhibition is shown to function in the intact cell, and evidence is presented which identifies the primary site of feedback control as a step leading to the formation of carbamylaspartate.

## MATERIALS AND METHODS

**Chemicals.** All radiolabeled chemicals, except [$^{14}$C]carbamylaspartate, and Aquasol (liquid scintillation cocktail) were purchased from New England Nuclear Corporation. The [ureido-$^{14}$C]carbamylaspartate was synthesized from [$^{14}$C]KCNO according to

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[3] Abbreviations: ACTase: aspartate carbamyltransferase; CPSase: glutamine-dependent carbamylphosphate synthetase; OPRTase: orotate phosphoribosyltransferase; ODCase: orotidine-5′-phosphate decarboxylase; ΣUMP: sum of the uridine nucleotides converted to UMP by acid hydrolysis; PRPP: 5-phosphoribosyl-1-pyrophosphate; DHOase: dihydroorotase; DHODHase: dihydroorotate dehydrogenase; OCTase: ornithine carbamyltransferase.

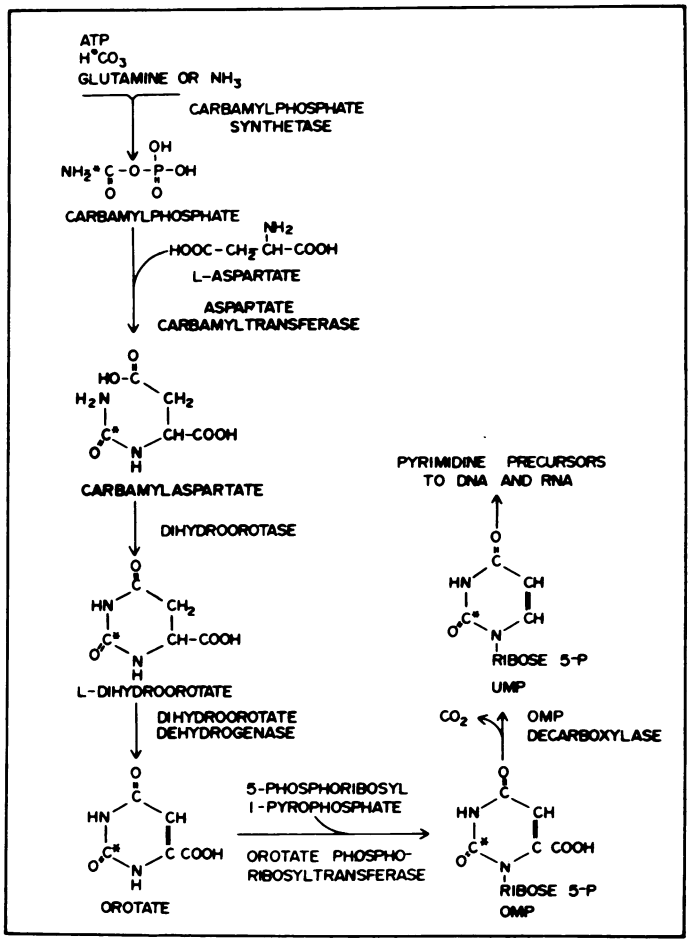


FIG. 1. Orotic acid pathway for *de novo* biosynthesis of pyrimidines. *Designates location of radiocarbon when [$^{14}$C]$NaHCO_3$ is employed as the precursor.

the method of Nyc and Mitchell (21). Mineral salts for modified Shive's (1) nutrient solution were of analytical reagent quality and were purchased from Fisher Scientific Company. All other chemicals were purchased from Sigma Chemical Company.

**Determination of Radioisotope Content.** In order to determine the content of radioisotope, samples were prepared as described in the text. In all cases, the samples were subsequently diluted with 6.5 ml Aquasol, chilled to 4 C, and the content of radioisotope measured by using a Nuclear-Chicago 300 liquid scintillation spectrometer.

**Plant Material.** Seeds of summer squash (*Cucurbita pepo* L. cv. Early Prolific Straightneck) courtesy of the Joseph Harris Co., Inc., were imbibed in distilled $H_2O$ for 24 h at room temperature. 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 $H_2O$. The covered box was placed in a growth chamber, where the seeds germinated in the dark for 48 h at 31 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 moistened paper towels until weighing was completed. About 40 roots were needed to obtain 750 mg, wet weight, of tissue.

## INCORPORATION OF RADIOLABELED PRECURSORS INTO OROTIC ACID AND URIDINE NUCLEOTIDES

**Preincubation.** Aliquots of excised roots were routinely preincubated for 2 h at 31 C in modified Shive's nutrient solution adjusted to pH 7.4. At the end of the preincubation period, the nutrient solution was decanted and discarded and the roots immediately transferred to fresh Shive's nutrient solution for incubation.

**Incubation.** The Shive's nutrient solution containing preincubated roots was supplemented with one of the following radiolabeled precursors at the concentration and specific radioactivity indicated: 10 mM $NaH^{14}CO_3$, 1,650 dpm/nmol; 5 mM [ureido-$^{14}$C]carbamylaspartate, 120 dpm/nmol; 2 mM [2-$^{14}$C] or [6-$^{14}$C] orotic acid, 75 dpm/nmol; 2 mM [carboxyl-$^{14}$C]orotic acid, 15 dpm/nmol. The reaction mixtures were incubated for 3 h at 31 C in a shaking water bath. When measuring the incorporation of precursor into orotic acid, 750 mg of excised roots were incubated in 20 ml Shive's nutrient solution, and 6-azauridine was routinely added at a reaction concentration of 0.5 mM to inhibit the conversion of orotic acid to UMP. Due to the expense of carrier UMP, measurements of the incorporation of percursor into ΣUMP routinely employed 150 mg excised roots incubated in 5 ml Shive's nutrient solution, with 6-azauridine omitted from the incubation mixture. The incubations were carried out in Erlenmeyer flasks sealed with rubber stoppers fitted with a plastic center well (Kontes Glassware, Vineland, N.J.) 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 ice-cold $HClO_4$ into the main chamber to a final concentration of 0.25 to 0.5 N. When $NaH^{14}CO_3$ was employed as a precursor, the amount of radioactivity used in a typical experiment was too great for disposal by air or sewage. When using $NaH^{14}CO_3$, the unreacted radioisotope was allowed to distill from the acidified incubation mixture into the KOH in the center well for an additional 10-min incubation at 31 C, and the trapped $^{14}CO_2$ was disposed of as solid waste through Interex Corporation, Natick, Mass. In all cases the contents of the main chamber were homogenized with a Polytron (PCU-2, Brinkmann Instruments), and the insoluble material removed by centrifugation at 10,000*g* for 10 min at 0 C. When orotic acid was to be isolated, the acid-soluble supernatant fraction was neutralized with 4 N KOH, and the resulting precipitate of $KClO_4$ was removed by centrifugation. The orotic acid synthesized from radiolabeled precursors during the incubation period was isolated from the neutralized acid-soluble fraction by co-crystallization with carrier monosodium orotate (33). When the incorporation of radiolabeled precursors into ΣUMP was to be determined, the uridine nucleotides were converted by acid hydrolysis to UMP by heating the acid-soluble supernatant fraction at 100 C for 1 h prior to neutralization with KOH. The neutralized hydrolysate was diluted with 2 parts of ethanol, and the uridine nucleotides synthesized from radiolabeled precursors were isolated as UMP by co-crystallization with carrier (6). Metabolites isolated by co-crystallization with carrier were always recrystallized to a constant specific radioactivity. When [carboxyl-$^{14}$C]orotic acid was employed as the precursor, 0.3 ml of 20% KOH (w/v) was injected into the plastic center well at the initiation of the incubation period, and the $^{14}CO_2$ generated during the incubation period was distilled into the KOH by terminating the reaction with acid and incubating the acidified reaction mixture for an additional 30 min at 31 C. The plastic center well and its contents were transferred to a scintillation vial and rinsed with 1.7 ml $H_2O$ to determine the content of radioisotope.

## IDENTITY OF THE METABOLITE OF $NAH^{14}CO_3$ THAT CO-CRYSTALLIZES WITH CARRIER UMP

The identity of the metabolite of $NaH^{14}CO_3$ that co-crystallized with carrier UMP was determined by descending paper chromatography employing isobutyric acid-0.5 N $NH_4OH$ (pH 3.6) (10/6, v/v) (5) as the developing solvent. Marker UMP, run in parallel with the sample, was located with an UV light and the corresponding area from the chromatogram of the sample was eluted with 5

ml $H_2O$ to determine its content of UMP and radioisotope. The content of UMP was assessed by measuring the *A* at 260 nm of a 1:60 dilution in 0.01 N HCl.

### IDENTITY OF THE METABOLITE OF $NAH^{14}CO_3$ THAT CO-CRYSTALLIZES WITH CARRIER OROTIC ACID

The metabolite of $NaH^{14}CO_3$ that co-crystallized with carrier orotate was identified by enzymic removal. The neutralized acid-soluble fraction obtained after incubation of the excised roots with $NaH^{14}CO_3$ was freeze-dried, reconstituted in 6.5 ml of water, and divided into three aliquots of 2 ml each. The first aliquot was diluted with 0.8 ml of water, the second with an equal volume of water containing 3 units of the mixed enzymes OPRTase and ODCase (from yeast, 1 unit catalyzes the conversion of 1 μmol of orotic acid to UMP per h at 25 C); and the third aliquot was diluted with an equal volume of water containing 3 units of the mixed enzymes and PRPP, 5.0 mM, a co-substrate in the conversion of orotic acid to UMP. After incubation for 4 h at 25 C, the reaction was terminated by transfer to a boiling water bath for 5 min. The precipitated protein was removed by centrifugation and the supernatant fluid was diluted to 25 ml with $H_2O$; 200 mg of carrier monosodium orotate was dissolved with heat and the orotate was allowed to crystallize as described above.

### DETECTION OF THE ENZYMIC ACTIVITIES OF THE OROTATE PATHWAY IN CELL-FREE EXTRACTS

Enzymic activities were measured in cell-free extracts prepared from excised roots. The roots were homogenized with a Duall all-glass conical tissue grinder (Kontes Glassware) in the specified homogenizing medium. The pH values of the various reagents employed in the enzyme assays were adjusted with HCl or KOH to the pH of the buffer employed in each assay. All assays were based upon measurement of the incorporation of $^{14}C$-labeled precursor into product.

**CPSase.** The activity of CPSase was measured in the presence of excess ornithine and commercial ornithine carbamyltransferase to convert all of the carbamylphosphate synthesized by the plant enzyme to citrulline. The following conditions for the extraction and assay of the enzyme were based upon the procedures of Ong and Jackson (26) and O'Neal and Naylor (24). 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) dimethylsulfoxide, 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; 10 mM L-ornithine; 2.3 units ornithine carbamyltransferase (Sigma), (1 unit catalyzes the formation of 1 μmol of citrulline/min at 37 C); 20 mM $NaH^{14}CO_3$, (1,100 dpm/nmol); and 0.5 ml of enzyme preparation. After 30-min incubation at 31 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. An aliquot of 2.5 ml of $H_2O$ containing 250 mg of carrier citrulline was added to the supernatant fluid, followed by the addition of 6 ml ethanol-isopropyl alcohol (1:1, v/v). Any resulting precipitate was dissolved with heat, and the [$^{14}C$]citrulline and carrier were allowed to co-crystallize as the solution cooled slowly to 4 C. The crystals were collected by suction filtration, washed with cold ethanol-isopropyl alcohol (1:1, v/v), and dried at 90 C to a constant weight. An aliquot of 10 mg was dissolved in 2 ml of $H_2O$ to determine the content of radioisotope. The citrulline was recrystallized to a constant specific radioactivity from $H_2O$-ethanol-isopropyl alcohol (1:1:1, v/v/v). The reliability of this procedure for isolating citrulline from the reaction mixture was established by recovering known amounts of commercial [ureido-$^{14}C$]citrulline from the complete reaction mixture at zero time. The results were confirmed by chromatography using Whatman No. 3 paper developed with 95% ethanol-1 M ammonium acetate (pH 7.5) 75:30 (v/v) (5). The [$^{14}C$]citrulline applied was located alongside a citrulline standard detected with 0.4% ninhydrin in 1-butanol. The spot containing [$^{14}C$]citrulline was eluted with 2 ml of $H_2O$ to measure its content of radioisotope.

**ACTase.** The assay procedure for measuring the activity of ACTase depends upon the lability of precursor, but not product, to acid; [$^{14}C$]carbamylphosphate is driven off as $^{14}CO_2$ under acidic conditions, whereas [$^{14}C$]carbamylaspartate is acid-stable (17). A homogenate of 1 g of excised roots in 5 ml of 0.1 M $KHCO_3$ was centrifuged at 10,000*g* at 0 C for 20 min, and the supernatant fluid 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; 16 mM [$^{14}C$]carbamylphosphate, 4.0 dpm/nmol; 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 evaporated to dryness over a boiling water bath. The residue, containing the acid-stable carbamylaspartate, was extracted with 2 ml of $H_2O$, and the content of acid-stable radioactivity was determined.

**DHOase and DHODHase.** The combined activities of these enzymes were detected by measuring the incorporation of [ureido-$^{14}C$]carbamylaspartate into [$^{14}C$]orotic acid. A homogenate of 1 g of excised roots in 5 ml of 50 mM phosphate buffer (pH 7.4), served as the source of the enzymes. The combined enzymic activities were demonstrated in a reaction mixture of 2-ml volume containing: 15 mM [$^{14}C$]carbamylaspartate, 83 dpm/nmol; 2 mM phosphate buffer (pH 7.4); and 1.0 ml of enzyme preparation. After 1-h incubation at 31 C, the reaction was stopped with 0.5 ml of 1.5 N $HClO_4$, and the precipitated protein was removed by centrifugation. The supernatant liquid was neutralized with KOH, the resulting precipitate of $KClO_4$ was removed by a second centrifugation, water was added to bring the volume to 25 ml, and 200 mg of carrier monosodium orotate was dissolved with heat. The [$^{14}C$]orotic acid produced by the combined activities of DHOase and DHODHase was allowed to co-crystallize with carrier orotate, and the resulting crystals were recrystallized to a constant specific radioactivity as described above. An aliquot was incubated in a reaction volume of 4 ml at pH 8 with 20 units of OPRTase and ODCase (yeast), while two identical aliquots were treated with OPRTase and ODCase plus PRPP, (20 μmol added at the initiation of the incubation and again after 2 h). After 4 h of incubation, the reactions were terminated by transfer to a boiling water bath, and the reactants and products contained in the supernatant fluid were separated by paper chromatography using 95% ethanol-1 M ammonium acetate (pH 7.5) (75:30, v/v) (5) as the developing solvent. The migration of standards of orotic acid and UMP was determined with an UV light, and corresponding zones of the sample chromatograms were eluted with 5 ml of $H_2O$. Aliquots of the eluates were counted to determine their content of radioisotope.

**OPRTase and ODCase.** The combined activities of these two enzymes were detected by the method of Rubin *et al.* (30), which measures the generation of $^{14}CO_2$ from [carboxyl-$^{14}C$]orotic acid. Homogenates containing 1 to 2 g of excised roots in 5 ml of water were centrifuged at 10,000*g* at 0 C for 20 min and the supernatant fluids used as the source of enzyme. The complete incubation mixture contained in a 1.5 ml volume: 50 mM Tris buffer, (pH 7.4); 3 mM $MgCl_2$; 0.5 mM [carboxyl-$^{14}C$]orotic acid, 592 dpm/

nmol; 1 mM PRPP; and 0.2 ml of enzyme preparation. The reaction vessels were sealed immediately with a rubber stopper fitted with a plastic center well containing 0.3 ml of 20% KOH and a filter paper wick. Following incubation for 30 min at 31 C, the reaction was terminated by injection of 2.5 ml of 1.5 N $HClO_4$, and the $^{14}CO_2$ generated was allowed to distill into the KOH for an additional 10 min at 31 C. The plastic center well and its contents were then transferred to a scintillation vial, rinsed with 1.7 ml of water, and the content of radioisotope was determined.

## RESULTS

**Demonstration of the *de novo* Biosynthesis of Pyrimidine Nucleotides.** The occurrence of the complete orotate pathway for the *de novo* biosynthesis of pyrimidine nucleotides in the intact cells of excised squash roots was indicated by demonstration of the incorporation of $NaH^{14}CO_3$ into orotic acid and ΣUMP, and by the observation that 6-azauridine blocked the incorporation of $NaH^{14}CO_3$ into ΣUMP while causing the concomitant accumulation of [$^{14}$C]orotic acid (Table I). It had been shown by others that 6-azauridine prevents the conversion of OMP to UMP in animal (11) and plant (28) tissues, with the consequent accumulation of orotic acid, orotidine, and OMP (29). Additional evidence that pyrimidine nucleotide biosynthesis proceeds via the orotate pathway in *C. pepo* was provided by demonstration of the incorporation of radiolabeled intermediates of this pathway into orotic acid and ΣUMP. Roots excised from 2-day-old, dark-germinated squash seeds were found to utilize carbamylaspartate and orotic acid, as well as bicarbonate, as precursors of uridine nucleotides, and in each case the incorporation was inhibited by the addition of 6-azauridine (Table II). Optimal conditions for precursor incorporation were determined for $NaHCO_3$ only. Significance should not be attached to the variations in sensitivity of precursor incorporation to 6-azauridine. Such variations probably arise from differences in the rate of appearance of label in OMP relative to the rate of conversion of 6-azauridine to 6-azaUMP; it is the 6-azaUMP which inhibits OMP decarboxylase.

The reliability of our procedure for the isolation of labeled UMP and orotic acid by co-crystallization with carrier was verified in separate experiments employing chromatographic and enzymic methods of analysis. The radiolabeled metabolite of $NaH^{14}CO_3$ isolated by co-crystallization with carrier UMP was shown to co-chromatograph with commercial UMP. In addition, chromatography of the carrier UMP containing the metabolite of $NaH^{14}CO_3$ did not result in a change in its specific radioactivity; the specific radioactivity of the UMP from two different incubation mixtures was 14,135 dpm/$A_{260}$ and 11,383 dpm/$A_{260}$ before chromatography, and 14,218 dpm/$A_{260}$ and 11,076 dpm/$A_{260}$, respectively, after chromatography.

Table I. *Incorporation of $NaH^{14}CO_3$ into Orotic Acid and ΣUMP*

The data represent nmol precursor incorporated per g tissue in 3 h. Values are shown as averages ± SE with the number of observations given in parentheses.

| Conditions | Orotic Acid | ΣUMP |
|---|---|---|
| No 6-Azauridine | 21 ± 4 (19) | 144 ± 10 (15) |
| 6-Azauridine, 0.5 mM | 84 ± 6 (34) | 27 ± 3 (3) |

Table II. *Incorporation of Precursors of Orotate Pathway into ΣUMP in Intact Cells of Squash Roots*

Conditions for the incorporation of 10 mM [$^{14}$C]$NaHCO_3$ (37.5 μCi) were optimal; no attempt was made to determine the optimal conditions for the incorporation of 5 mM [$^{14}$C]carbamylaspartate (1.4 μCi), or 1 mM [$^{14}C_6$]orotic acid (0.33 μCi).

| $^{14}$C-Labeled Precursor | Incorporation into ΣUMP[a] | Inhibition by 0.5 mM 6-Azauridine (%) |
|---|---|---|
| $NaHCO_3$ | 144 ± 10 (15) | 77 ± 1 (3) |
| Carbamylaspartate | 224 ± 31 (4) | 47 ± 10 (4) |
| Orotic acid | 496 ± 10 (7) | 41 ± 2 (6) |

[a] Nanomol precursor incorporated per g tissue in 3 h. Values are shown as averages ± SE with number of observations given in parentheses.

Table III. *Identity of Metabolite Synthesized from $NaH^{14}CO_3$ That Co-Crystallizes with Carrier Orotate*

| Treatment Prior to Co-Crystallization | Radioactivity Isolated with Carrier Monosodium Orotate | |
|---|---|---|
| | *cpm/10 mg* | *% control* |
| None (control) | 920 | 100 |
| Mixed enzymes (OPRTase and ODCase) | 949 | 103 |
| Mixed enzymes plus PRPP | 105 | 11 |

The identity of the metabolite of $NaH^{14}CO_3$ isolated by co-crystallization with carrier orotate was established to be [$^{14}$C]orotic acid by its enzymic removal prior to co-crystallization. Incubation of the neutralized, acid-soluble fraction of the reaction mixture with OPRTase, ODCase, and PRPP catalyzes the condensation of orotic acid with PRPP to form OMP, and the conversion of OMP to UMP. Subsequent co-crystallization of metabolites with carrier orotic acid showed a loss of 89% of the radioisotope isolated from an equal aliquot not subjected to enzymic treatment (Table III). This loss, and its dependency on PRPP, establishes the identity of the metabolite of $NaH^{14}CO_3$ co-crystallizing with carrier orotate as [$^{14}$C]orotic acid.

Evidence that the observed incorporation represents the activity of the orotate pathway in intact cells rather than a small population of ruptured cells was obtained by comparing the activity of excised roots with that of a cell-free extract prepared from the same amount of tissue; virtually no incorporation occurred with the cell-free extract.

Finally, evidence of the occurrence of the complete orotic acid pathway in *C. pepo*, obtained in the studies with intact cells described above, was corroborated by assays of the component enzymic activities in cell-free extracts. All assays were performed with cell-free extracts prepared from 2-day old, dark-germinated squash roots. Assay conditions were those reported to be optimal for these enzymes from other tissue sources; no attempts were made to optimize the assay conditions for the enzymes of squash roots. The enzymic activity of carbamylphosphate synthetase (CPSase) was coupled with commercial ornithine carbamyltransferase (OCTase), added in excess. Assessment of the activity of CPSase by measuring the incorporation of $NaH^{14}CO_3$ into acid-stable product was unreliable because of the formation of radiolabeled acid-stable compounds other than citrulline; thus, [$^{14}$C]-citrulline was isolated from the incubation mixture by co-crystallization with carrier (see under "Materials and Methods"). The reliability of our method for isolating citrulline from the reaction mixture was demonstrated by the isolation of virtually all of the radiolabel from chromatographically pure commercial [ureido-$^{14}$C]citrulline from identical reaction mixtures. In addition, maximum formation of the [$^{14}$C] $^{14}$C metabolite of $NaH^{14}CO_3$ which co-crystallized with carrier citrulline was dependent upon the inclusion of the cell-free extract, OCTase and ornithine, ornithine, glutamine, and ATP, in the reaction mixture (Table IV). The activities of CPSase, ACTase, DHOase in combination with DHODHase, and OPRTase in combination with ODCase in cell-free extracts of squash roots are listed in Tables IV and V.

**Determination of Optimal Conditions for Measuring the Activity of the Orotate Pathway in Intact Cells.** Due to the expense of carrier UMP, optimal conditions for measuring the activity of the orotate pathway in intact cells were determined by measuring the incorporation of $NaH^{14}CO_3$ into orotic acid. Assays routinely employed 750 mg of excised roots suspended in 20 ml of Shive's

Table IV. *Carbamylphosphate Synthetase Activity*

The values are given as cpm/10 mg carrier citrulline used in the isolation of [$^{14}$C]citrulline by co-crystallization.

| Incubation Conditions | Incorporation of $NaH^{14}CO_3$ into Citrulline | | |
|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 |
| Complete (control) | 564 | 249 | 510 |
| Minus cell-free extract | | 36 | 64 |
| Minus ornithine and OCTase | 65 | 99 | 153 |
| Minus ornithine | | 164 | 339 |
| Minus ATP | 89 | 85 | 130 |
| Minus glutamine | 435 | 90 | 320 |

Table V. *Enzymic Activities of Cell-free Extracts of C. pepo Roots*

Assay conditions were those shown to be optimal by other investigators; no attempts were made to optimize conditions of assay for enzymes of *C. pepo*. ACTase was assayed at 22 C and the remaining enzymes at 31 C. The values given for ACTase and the combined activities of OPRTase and ODCase represent the total amount of product formed per reaction mixture. CPSase activity is given in Table IV.

| Assay Conditions: Additions or Deletions | ACTase | DHOase with DHODHase | OPRTase with ODCase |
|---|---|---|---|
| | *cpm* | *cpm* | *cpm* |
| None (complete) | 1,447 | 390[a] | 22,512 |
| Enzyme omitted | 434 | — | 440 |
| PRPP omitted | — | — | 398 |
| Pyrophosphate, 2 mM, added | — | — | 11,756 |
| Aspartate omitted | 588 | — | — |
| Blank[a] | — | 70 | — |

[a] The coupled activity of DHOase and DHODHase represents the amount of radioactivity contained in an aliquot of the [$^{14}$C]orotic acid isolated by co-crystallization with carrier and purified further by chromatography. The blank value is the result of an assay of an equal aliquot of crystals treated with OPRTase, ODCase, and PRPP prior to chromatographic purification; 80% of the radioactivity contained in the orotic acid peak co-chromatographed with UMP after treatment with OPRTase, ODCase, and PRPP.

nutrient solution. In experiments measuring incorporation into orotic acid, 6-azauridine was added at a reaction concentration of 0.5 mM; this concentration allowed maximal accumulation of orotic acid (Fig. 2) and also maximally inhibited generation of $^{14}CO_2$ from [carboxy-$^{14}$C]orotic acid. The optimal concentration of $NaH^{14}CO_3$ was about 10 mM (Fig. 3). These optimal concentrations were used throughout. Neither the addition of 0.3 M glucose nor the substitution of Krebs Improved Ringer II solution (33) for Shive's nutrient solution stimulated incorporation of $NaH^{14}CO_3$ into orotic acid. Preincubation of excised squash roots for 2 h at 31 C in Shive's solution significantly enhanced incorporation of $NaH^{14}CO_3$ into orotic acid during the subsequent incubation period. Inclusion of 6-azauridine during the preincubation period was without effect. Thus, all incorporation studies reported herein employed a 2-h preincubation period in Shive's solution alone, followed by a second incubation period of 3 h in fresh Shive's solution supplemented with labeled precursor and other components, as indicated.

The possibility that the observed incorporation reflected the activity of fungal or bacterial contamination of the seedlings is considered unlikely since no growth of microorganisms was detected after incubation of aliquots of excised roots in the dark for 24 h at 31 C on potato dextrose agar.

Having verified the reliability of our procedures for measuring pyrimidine biosynthesis in intact cells, and having established optimal conditions for measuring the incorporation of $NaH^{14}CO_3$ into orotic acid, we proceeded to determine whether we could detect end product inhibition of pyrimidine biosynthesis in these cells.

**End Product Inhibition of the Orotate Pathway in Intact Cells of Squash Roots.** Addition of uridine or cytidine to the incubation mixture inhibited the incorporation of $NaH^{14}CO_3$ into orotic acid and into ΣUMP. An extracellular concentration of only 0.5 mM was sufficient to produce the maximum inhibition (79–88%) of incorporation into orotic acid by either nucleoside (Fig. 4 and Table VI).

Reversibility of the inhibition by uridine was tested by transferring roots incubated with uridine for 3 h to a uridine-free medium and measuring the incorporation of $NaH^{14}CO_3$ into ΣUMP during a subsequent 3-h incubation period (Table VII).

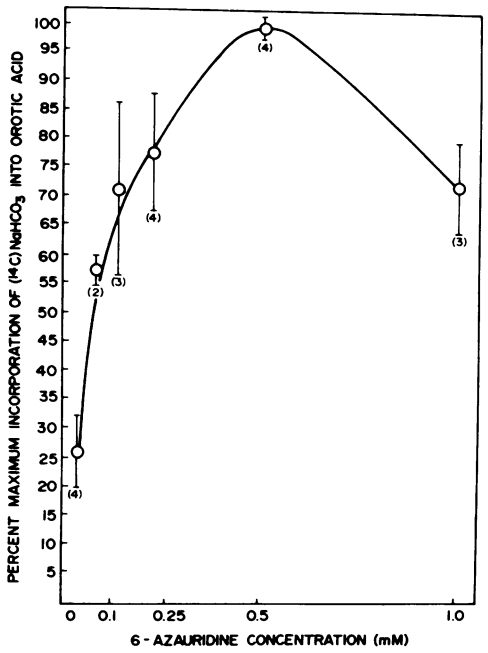


FIG. 2. Effect of 6-azauridine on accumulation of [$^{14}$C]orotic acid synthesized from $NaH^{14}CO_3$. Bars indicate standard error for each average value with the number of observations given in parentheses.

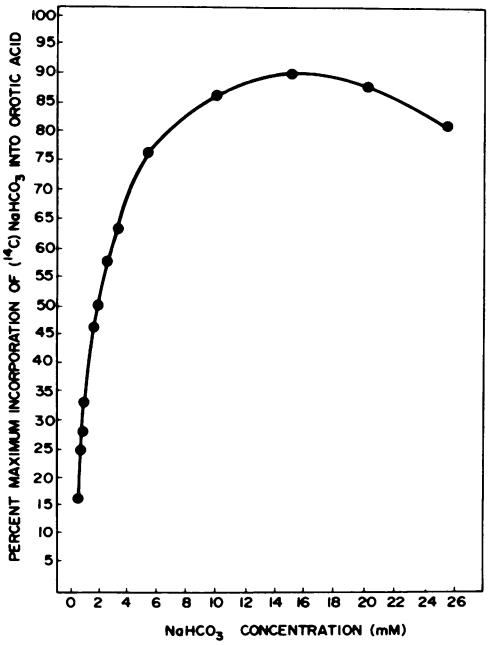


FIG. 3. Incorporation of $NaH^{14}CO_3$ into [$^{14}$C]orotic acid as a function of $NaHCO_3$ concentration. Curve represents pooled data from two to five assays at each concentration.

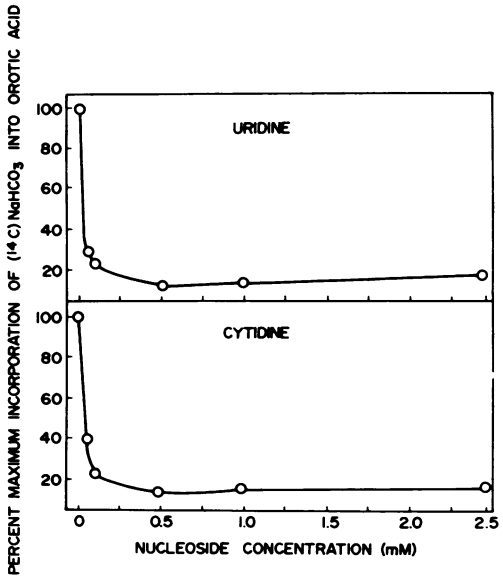


FIG. 4. Sensitivity of orotic acid synthesis to end product inhibition. Each value is expressed as a percentage of the incorporation observed in the absence of added nucleoside.

Table VI. *End Product Inhibition of Incorporation of $NaH^{14}CO_3$ into Orotic Acid and ΣUMP*

| Additions | Incorporation of $NaH^{14}CO_3$ into Products | |
|---|---|---|
| | Orotic acid | ΣUMP |
| | *(% inhibition)*[a] | |
| Uridine, 0.5 mM | 88 ± 1 (3) | 82 ± 2 (8) |
| Cytidine, 0.5 mM | 85 ± 1 (3) | 79 ± 1 (4) |

[a] The degree of inhibition was calculated from the corresponding value obtained in the absence of added nucleoside for each assay, and is presented as an average value ± SE with the number of observations in parentheses. The absolute values obtained in the absence of added nucleoside are included in the data presented in Table I.

Table VII. *Recovery from End Product Inhibition*

| Assay No. | Incubation Conditions | | Incorporation of $NaH^{14}CO_3$ into ΣUMP[a] | |
|---|---|---|---|---|
| | 0-3 h | 3-6 h | 0-3 h | 3-6 h |
| 1 | $NaH^{14}CO_3$ | | 100 | |
| 2 | $NaH^{14}CO_3$ + uridine | | 20 | |
| 3 | No additions | $NaH^{14}CO_3$ | | 51 |
| 4 | Uridine | $NaH^{14}CO_3$ + uridine | | 10 |
| 5 | Uridine | $NaH^{14}CO_3$ (−uridine) | | 43 |

[a] Values are expressed as per cent of control; control value is 154 nmol $NaH^{14}CO_3$ incorporated into ΣUMP during the first 3 h of incubation in the absence of uridine. Final reaction concentration for $NaH^{14}CO_3$ was 10 mM and for uridine, 0.5 mM.

The prolonged incubation time reduced the uninhibited rate of incorporation during the second 3-h incubation period to 51% of the rate observed during the first 3-h period. If uridine was included in the reaction mixture throughout the 6 h of incubation, the degree of inhibition during the second 3-h incubation period was essentially the same as that observed during the first 3 h (compare 51 declining to 10 with 100 declining to 20, respectively). Transfer of the roots to a uridine-free medium after the first 3-h incubation period restored over 80% of the *de novo* activity during the second incubation period (compare 43 with 51). Thus, the inhibition by uridine was readily reversible. These results demonstrate the operation of end product inhibition as a regulatory mechanism governing the activity of the orotate pathway in intact root cells. Although this regulatory mechanism was readily detected by the addition of uridine to the incubation mixture, data from studies by others leave little reason to doubt that the actual inhibitor is a pyrimidine nucleotide synthesized from uridine; evidence that squash roots are able to convert uridine to its nucleotides is given in Table IX.

**Site of End Product Inhibition by Added Uridine or Its Metabolites.** By employing various radiolabeled precursors of UMP, we determined which enzymes of the orotate pathway were sensitive to end product inhibition. Uridine or its metabolites inhibited the incorporation of $NaH^{14}CO_3$, but not [$^{14}$C]carbamylaspartate or [$^{14}$C]orotic acid, into ΣUMP (Table VIII). These results show end product inhibition to occur at one or both of the enzymic reactions leading to carbamylaspartate formation, and exclude the remaining enzymes of the pathway as additional sites of physiological significance in the regulation of pyrimidine biosynthesis by feedback control.

**Consideration of Alternative Pathways for Pyrimidine Biosynthesis.** Alternative pathways have been suggested for the synthesis of UMP from orotic acid in plants. In addition to the conversion of orotic acid to UMP via OMP (Fig. 1), Buchowicz and Reifer (4) have proposed the direct decarboxylation of orotic acid to uracil, followed by the incorporation of uracil into UMP through the salvage enzymes. Further studies in their laboratory provided evidence that the synthesis of UMP from orotic acid might also proceed through OMP, but indirectly, *i.e.* through the conversion of OMP to orotidine followed by decarboxylation of orotidine to uridine and salvage of uridine to UMP (34). Lastly, Buchowicz and Lesniewsak (3) have reported results which they interpreted to indicate direct conversion of orotic acid to uridine by an unknown mechanism, thus offering a fourth route by which orotic acid might be converted to UMP in plants. We investigated the occurrence of these alternative pathways to ascertain their relative contribution, if any, to UMP synthesis in *C. pepo*.

We could not detect enzyme-catalyzed $^{14}CO_2$ generation from [carboxyl-$^{14}$C]orotic acid in cell-free extracts unless PRPP was added to the reaction mixture, and pyrophosphate inhibited the PRPP-dependent $^{14}CO_2$ generation (Table V). These results are difficult to reconcile with a direct conversion of orotic acid to either uracil or uridine, while they are consistent with the reversible phosphoribosylation of orotic acid to OMP + PPi prior to decarboxylation. In studies employing intact cells of squash roots, neither uracil, uridine, nor orotidine diluted the incorporation of ring-labeled orotic acid into uridine nucleotides (Table IX). The ability of intact cells of *C. pepo* roots to utilize uracil and uridine provided exogenously was verified by direct measurements of the incorporation of these radiolabeled pyrimidines into uridine nucleotides (Table IX). The failure of either uracil or uridine to dilute the incorporation of ring-labeled orotic acid into uridine nucleotides is also contrary to their putative role as intermediates in UMP synthesis from orotic acid. The additional observation that orotidine is without influence on the incorporation of ring-labeled orotic acid into uridine nucleotides argues against the occurrence in squash roots of a pathway for the conversion of orotic acid to UMP via the sequential formation of OMP, oroti-

Table VIII. *Localization of Site of End Product Inhibition*

| $^{14}$C-labeled Precursor | Incorporation of Precursor into ΣUMP[a] | | Inhibition |
|---|---|---|---|
| | −Uridine | +Uridine | |
| | | | % |
| $NaHCO_3$ | 137 ± 23 | 24 ± 2 | 82 |
| Carbamylaspartate | 224 ± 31 | 207 ± 30 | 8 |
| Orotic acid | 496 ± 10 | 547 ± 6 | −10 |

[a] Values are averages ± SE ($N$ = 4-8) in nmol of precursor incorporated into ΣUMP per g tissue in 3 h. The conditions are given in the legend to Table II. Concentration of uridine was 0.5 mM.

Table IX. *Influence of Uracil, Uridine, and Orotidine on Incorporation of [$^{14}C_6$]Orotic Acid into ΣUMP*

Each assay was performed in duplicate and the values listed are the averages. Average control value for the two experiments was 447 nmol 2 mM [$^{14}C_6$]orotic acid, 146 dpm/nmol, incorporated into ΣUMP/g tissue in 3 h. In four separate experiments designed to test the capacity of roots to utilize exogenously provided 5 mM [$^{14}$C]uridine 270 dpm/nmol, and 5 mM [$^{14}$C]uracil, 114 dpm/nmol, the average incorporation of these precursors into ΣUMP was 429 ± 58 nmol and 100 ± 31 nmol/g tissue in 3 h, respectively.

| Addition | cpm/150 mg carrier UMP | |
|---|---|---|
| | Exp. 1 | Exp. 2 |
| None (control) | 1774 | 1558 |
| Uracil, 10 mM | 1652 | 1681 |
| Uridine, 10 mM | 2024 | 1636 |
| Orotidine, 10 mM | 1872 | 1538 |

dine, and uridine (Table IX). Thus, we found no evidence of any of these alternative routes for UMP synthesis in *C. pepo*.

Other workers have also been unable to detect evidence of these alternate routes of UMP synthesis from orotic acid in plants. Ross (29) showed that generation of $^{14}CO_2$ from [carboxyl-$^{14}$C]orotic acid in bean leaf homogenates requires the addition of PRPP, and Ashihara (2) obtained similar results with extracts of black gram seedlings and wheat seedlings. In assays employing bean leaf homogenates, Wolcott and Ross (35) also found that 6-azauridine inhibited generation of $^{14}CO_2$ from [carboxyl-$^{14}$C]orotic acid and caused the accumulation of $^{14}$C-labeled orotidine and OMP. Ross (29) obtained similar results in a system employing leaf discs of both bean and cocklebur. Given that 6-azauridine inhibits ODCase (11, 28), the accumulation of these pyrimidine compounds argues that the activity of ODCase is necessary for UMP synthesis from orotic acid. These reports and our own data suggest that the alternative pathways proposed for the conversion of orotic acid to UMP are of insufficient activity or too limited distribution to contribute significantly toward the biosynthesis of pyrimidine nucleotides in plants in general.

## DISCUSSION

Squash roots possess the full complement of enzymes for the *de novo* biosynthesis of uridine nucleotides. The occurrence of the complete orotate pathway was established by demonstrating the incorporation of several radiolabeled precursors, including $NaH^{14}CO_3$, into both orotic acid and uridine nucleotides. In addition, the activities of the component enzymes of the pathway were detected in cell-free extracts.

The capacity of 2-day-old squash roots to synthesize pyrimidines *de novo* was assessed by measuring the activity of the orotate pathway in intact cells under optimal conditions; 144 ± 10 nmol ($\bar{X}$ ± SE, N = 15) $NaH^{14}CO_3$ were incorporated into uridine nucleotides per g tissue during 3 h of incubation. By way of comparison, the activity of squash roots was about double that observed in slices of the rat spleen by the same assay procedure (6).

The incorporation of $NaH^{14}CO_3$ into orotic acid and uridine nucleotides was inhibited by the addition of uridine or cytidine to the incubation medium, and the inhibition by added uridine was readily reversible upon transfer of the roots to a uridine-free medium. These results demonstrate end product inhibition of pyrimidine biosynthesis *de novo* in the intact plant cell. In an attempt to localize the site of inhibition, the influence of uridine on the incorporation of sequential intermediates of the pathway into uridine nucleotides was measured. Uridine 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. These results show regulation of pyrimidine biosynthesis by feedback inhibition to occur at one or both of the enzymic reactions leasing to synthesis of carbamylaspartate. However, we could not distinguish between regulation at CPSase and ACTase. Regulation at either enzyme would result in inhibition of the incorporation of $NaH^{14}CO_3$ into ΣUMP. We attempted to detect feedback inhibition at ACTase in the intact cell by substituting 10 mM [$^{14}$C]carbamylphosphate (5 μCi) for $NaH^{14}CO_3$, but the incorporation of [$^{14}$C]carbamylphosphate into ΣUMP was too low to be considered reliable. The low incorporation of carbamylphosphate is most likely the result of its inability to penetrate the cell membrane. In addition, the incorporation observed was ambiguous because it could also have arisen from incorporation of $^{14}CO_2$ generated by the spontaneous or enzyme-catalyzed (7, 10, 12) breakdown of [$^{14}$C]carbamylphosphate. We were unable to determine whether ACTase is a physiologically important regulatory site, but we can state with reasonable certainty that one or both of the first two reactions in the orotate pathway, those catalyzed by CPSase and ACTase, are the only reactions of physiological importance in the regulation of pyrimidine biosynthesis by end product inhibition in the intact cells of squash roots.

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