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Application of Commercial Enzymes to Measure the Activity of the Arginine Pathway–Urea Cycle in Intact Cells[1]

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The activity of the complete arginine pathway–urea cycle was assessed in intact plant cells by employing the commercial enzymes arginase (EC 3.5.3.1) and urease (EC 3.5.1.5) to determine the amount of $NaH^{14}CO_3$ incorporated into [*guanido*-^{14}C]arginine and/or into [^{14}C]urea during a 3-h labeling period. Recovery of [*guanido*-^{14}C]arginine was linear from 5 to 1000 nmol/g tissue and averaged 80 ± 5% (mean ± SE, N = 3). The procedure is reliable, inexpensive, well suited to the simultaneous analysis of numerous samples, and significantly more sensitive than existing methods. The method is ideally suited for assessing the activity of the complete arginine biosynthetic pathway in intact cells. In addition, the method has the distinct advantage of providing simultaneous measurement of the amount of $NaH^{14}CO_3$ accumulating in arginine relative to the amount accumulating as urea. Evidence is presented demonstrating that both the activity of the arginine pathway and the relative amounts of [*guanido*-^{14}C]arginine and [^{14}C]urea synthesized from $NaH^{14}CO_3$ were influenced by changes in the level of ornithine, NH_4^+, or phosphorus available to plant tissues. © 1984 Academic Press, Inc.

KEY WORDS: arginine synthesis; urea cycle; plant systems.

It has been proposed (1,2) that in vascular plants, the activities of the orotic acid pathway for the *de novo* biosynthesis of pyrimidine nucleotides and the pathway for the *de novo* biosynthesis of arginine are regulated in a coordinated manner at carbamoylphosphate synthetase (EC 6.3.5.5), the only enzyme in vascular plants known to synthesize carbamoylphosphate (1–4). To investigate this possibility and to determine the manner by which carbamoylphosphate is allocated to these two pathways in plant cells of different physiological states, it was desirable to be able to assess the activity of both *de novo* pyrimidine and arginine biosynthetic pathways in intact plant cells by measuring the incorporation of $NaH^{14}CO_3$ into a metabolite of each pathway.

A method for assessing the capacity of intact plant cells to synthesize pyrimidine nucleotides *de novo* by measuring the incorporation of $NaH^{14}CO_3$ into orotic acid or total uridine nucleotides (ΣUMP)[3] has previously been reported (5). Use of available cocrystallization procedures to isolate products of the urea cycle in plant cells, e.g., citrulline (5), arginine (C. J. Lovatt and A. H. Cheng, unpublished work), and urea (6), failed to isolate quantities of product significantly greater than background when $NaH^{14}CO_3$ was the precursor; TLC and paper chromatography procedures were also compromised when $NaH^{14}CO_3$ was used as the precursor. This situation was due to (i) the large number of radiolabeled metabolites synthesized from $NaH^{14}CO_3$ during the 3-h incubation period, and (ii) relatively low

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[3] Abbreviation used: ΣUMP, sum of the uridine nucleotides converted to UMP by acid hydrolysis (0.25–1 N $HClO_4$) for 1 h in a boiling-water bath.

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activity of the arginine pathway–urea cycle in plant tissues. For comparison, isolated hepatocytes incorporated 14.1 μmol $NaH^{14}CO_3$ (without added NH_4^+) into urea per gram wet weight of liver during a 1-h incubation period (6). The results of the present study with root and leaf tissues of a number of plant species demonstrated that the activity of the arginine pathway in the intact plant cell was 1000- to 3000-fold less.

In this communication, we present a simple, sensitive, and reliable method easily adapted to a large number of simultaneous assays in which the commercial enzymes arginase (EC 3.5.3.1) and urease (EC 3.5.1.5) are employed to quantify the amount of $NaH^{14}CO_3$ incorporated into arginine and/or urea by intact cells during a 3-h incubation period. The method is suitably flexible to permit the use of any radiolabeled precursor of the urea cycle which labels the guanidocarbon of arginine and has the distinct advantage of providing simultaneous measurement of the amount of ^{14}C-radiolabeled precursor incorporated into [*guanido*-^{14}C]arginine relative to the amount incorporated into [^{14}C]urea. We have observed that the incorporation of $NaH^{14}CO_3$ into arginine relative to the amount incorporated into urea was influenced by changes in the level of NH_4^+, ornithine, or phosphorus available to the tissue.

MATERIALS AND METHODS

Chemicals. Radiolabeled $NaH^{14}CO_3$ and [^{14}C]urea were purchased from ICN Pharmaceuticals, Inc., and [*guanido*-^{14}C]arginine was purchased from New England Nuclear. Liquiscint (liquid scintillation cocktail) was purchased from National Diagnostics. Mineral salts for modified Shive's and modified Hoagland's nutrient solutions were of analytical reagent quality from Fisher Scientific. 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 Company, 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 sheets of paper toweling, placed in a plastic box (33 × 23 × 9.5 cm), and moistened with 20 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 wt tissue. Seeds of *Citrus limon* cv. rough lemon, *Poncirus trifoliata* × *C. sinensis* cv. 'Carrizo' citrange and 'Troyer' citrange, and *P. trifoliata* cv. Australian trifoliate orange were germinated and grown in sterile sand containing 1 μg extractable P per gram, and watered twice a week with modified half-strength Hoagland's nutrient solution, pH 7.2, made with and without phosphorus as described by Hoagland and Arnon (7). The plants were maintained in a growth chamber under 16 h of light (310 μE m^{-2} s^{-1}) at 32°C and 8 h of dark at 21°C. The youngest fully expanded leaves were excised from eight 7-month-old seedlings (usually two to five leaves were excised per plant; midveins were removed). Leaf tissue was cut into 5 × 5-mm pieces, which were pooled and then weighed into 500-mg fresh wt samples which were maintained between sheets of moistened paper towels until all weighing was completed.

Leaf arginine content. Arginine content of leaves was determined in samples prepared according to Labanauskas and Handy (8) and analyzed on a Beckman 120C amino acid analyzer previously calibrated with commercial amino acid standards at 570 nm.

Incorporation of $NaH^{14}CO_3$ into acid-soluble products. Samples consisting of 150 mg fresh wt of roots excised from 2-day-old, dark-germinated squash seedlings or 500 mg

fresh wt leaves from phosphorus-sufficient and phosphorus-deficient citrus rootstock seedlings were routinely preincubated in 5 ml Shive's nutrient solution (9) without $(NH_4)_2SO_4$, pH 7.4, for 2 h at 30°C in a water bath–shaker. At the end of the preincubation period, the nutrient solution was decanted, and the tissue was immediately transferred to fresh Shive's nutrient solution supplemented with $NaH^{14}CO_3$ (10 mM, 150 μCi for roots and 5 mM, 37.5 μCi for leaf tissue) and any other additives to be tested 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. During both preincubation and incubation, all tissue was submerged but kept from clumping by constant shaking. Incorporations were carried out in a 25-ml Erlenmeyer flask sealed with a rubber stopper, fitted with a plastic center well (Kontes Glassware, Vineland, N. J.) containing a filter paper wick. At the end of the incorporation 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 6 N $HClO_4$ into the main chamber of the flask. The flasks were returned to the water bath–shaker for an additional 10 min to allow the unreacted $^{14}CO_2$ from the acidified incubation mixture to distill into the KOH in the center well. The center well, containing radioactive waste, was disposed of appropriately, and the contents of the main chamber of the flask were homogenized with a Polytron tissue homogenizer (PCU-2, Brinkman Instruments). The Polytron probe was rinsed with 2 ml of 1 N $HClO_4$ which was added to the homogenate. The insoluble material was removed by centrifugation at 10000*g* for 10 min at 0°C. A 2-ml aliquot was removed from the acid-soluble supernatant fraction for determination of the amount of $NaH^{14}CO_3$ into [*guanido*-^{14}C]arginine and into [^{14}C]urea, as described below. The remainder of the acid-soluble supernatant fraction was used to measure the incorporation of $NaH^{14}CO_3$ into orotic acid or ΣUMP (5).

Measurement of the amount of $NaH^{14}CO_3$ incorporated into [guanido-^{14}C]arginine and into [^{14}C]urea. The 2-ml aliquot removed from the acid-soluble supernatant solution was neutralized to pH 7.5 with KOH and made 50 mM with 17 mg $Ba(OH)_2$. The resulting precipitate was removed by centrifugation in a tabletop centrifuge (Dynac) at setting 70 for 10 min. The supernatant was made 100 mM glycine with 1 M glycine–NaOH buffer, pH 9.0, and brought to a final pH of 9.0 with NaOH. 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 (EC 3.5.3.1) (Sigma, from bovine liver; 1 unit catalyzes the conversion of 1.0 μmol of 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 (EC 3.5.1.5) (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). A second 1-ml sample was transferred to a second Erlenmeyer flask to which was added only urease. All flasks were sealed with a rubber stopper fitted with a plastic center well containing a filter paper wick and 0.3 ml of 20% KOH, and incubated in a water bath–shaker for 3 h at 37°C. The reaction was terminated by injecting $HClO_4$ into the main chamber of the flask to a final concentration of 1 N. Flasks were returned to the water bath–shaker for 30 min to allow the $^{14}CO_2$ released by urease to distill from the acidified incubation mixture into the KOH in the center well. An aliquot of 0.2 ml of 0.1 N $NaHCO_3$ was injected into the main chamber of the flask to flush any residual $^{14}CO_2$ from the incubation mixture. After 30 min at 37°C in the water bath–shaker, the center well and its contents were transferred to a scintillation vial 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 stan-

dardization system of quench analysis. A standard quench curve was determined by using a series of samples containing a known quantity of ^{14}C radioactivity and increasing amounts of a chemical quencher.

The reliability of this method to recover arginine was determined by adding aliquots of [^{14}C]arginine (previously purified by co-crystallization with carrier arginine, and thus, of known specific activity) to incubation mixtures of squash roots or citrus leaf tissue to give the equivalent of 1 to 1000 nmol/g tissue. Samples were prepared as described above, but incubated with unlabeled $NaHCO_3$. The reactions were terminated immediately by addition of $HClO_4$, and the content of [^{14}C]arginine was determined as described.

Calculation of the nanomoles of product synthesized. The nanomoles of product synthesized by the plant tissue from the radiolabeled precursor supplied in the incubation mixture is calculated as

$$\left(\frac{\text{dpm } ^{14}CO_2 \text{ in center well}}{\text{percentage recovery}}\right) \times \left(\frac{\text{dilution factor}}{\text{fraction of acid-soluble fraction assayed}}\right) \times \left(\frac{1}{\text{dpm/nmol precursor}}\right) \times \left(\frac{1000 \text{ mg}}{\text{mg tissue in the incubation mixture}}\right)$$

= nanomoles precursor incorporated into product per gram fresh weight tissue during the 3-h incubation.

The sample treated with both arginase and urease provides the measurement of the incorporation of the radiolabeled precursor into the sum of arginine plus urea and is useful in itself as an estimate of the activity of the *de novo* arginine biosynthetic pathway. The sample treated with urease only is the measurement of the incorporation of radiolabeled precursor into urea only. This value should be subtracted from the value obtained for the first sample to obtain the measurement of the incorporation of radiolabeled precursor into arginine.

RESULTS

The pH optimum for the combined activities of commercial arginase and urease was pH 9.0 (Fig. 1). At this pH, the recovery of [*guanido*-^{14}C]arginine was linear over a range of concentrations from 5 to 1000 nmol/g tissue (Fig. 2a). Average recovery was 80% over this range and, at any given arginine concentration, the standard error did not exceed ±5% (N = 3). Recovery of [^{14}C]urea was linear from 0 to 100 nmol/g tissue (Fig. 2b). While the recovery of arginine suggests that the recovery of urea would be linear over the same range of concentrations, we were specifically interested in recovery over the range of concentrations typical of those observed in plant tissues.

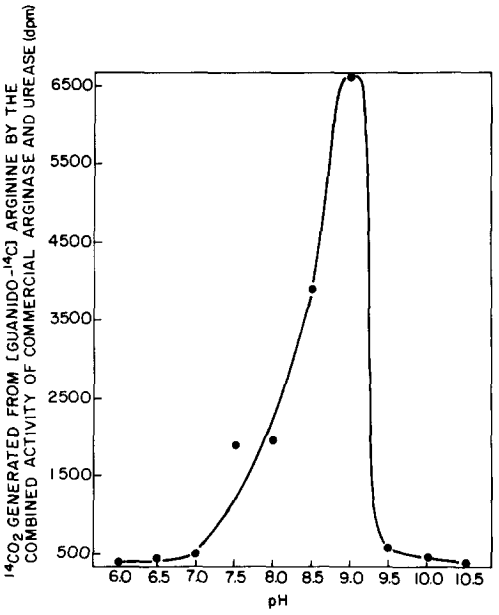

FIG. 1. Optimum pH for the combined activities of commercial arginase and urease with [*guanido*-^{14}C]arginine (0.3 μM, 0.1 μCi) as the substrate. The recovery of $^{14}CO_2$ generated by the combined enzymatic activities was carried out as described under Materials and Methods. The glycine–NaOH buffer was adjusted to cover the range of pH from 8.5 to 10.5 and was replaced with phosphate buffer for pH 6.0 to 8.0.

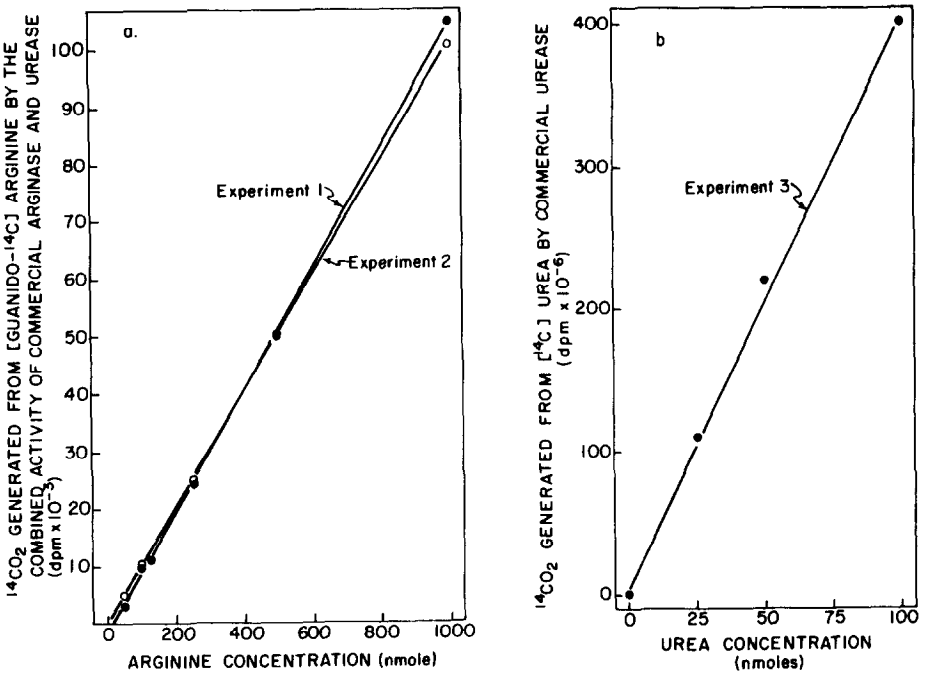

FIG. 2. Recovery of [*guanido*-^{14}C]arginine (a) and [^{14}C]urea (b) from the acid-soluble fraction of the incubation mixture as a function of concentration. In three separate experiments, [*guanido*-^{14}C]arginine or [^{14}C]urea of known concentration and specific activity was added to the incubation mixture containing 150 mg fresh wt intact roots, Shive's nutrient solution (pH 7.4), and unlabeled $NaHCO_3$ (10 mM). The reactions were immediately terminated by injecting 1 ml of 6 N $HClO_4$ into the main chamber of the flask. The amount of [*guanido*-^{14}C]arginine or [^{14}C]urea in each sample was determined as described under Materials and Methods.

The reproducibility of the method was demonstrated in three separate experiments employing roots excised from three different batches of 2-day-old, dark-germinated squash seedlings. Roots were incubated in the presence of exogenously supplied metabolites selected to provide a wide range of activities of the arginine pathway–urea cycle (Table 1).

The efficacy of this method for assessing the activity of the *de novo* arginine biosynthetic pathway was confirmed by providing evidence of the relationship between the activity of the arginine biosynthetic pathway and the arginine content of leaves of four different citrus rootstock cultivars. The activity of the arginine pathway was perturbed by withholding phosphorus from half of the seedlings during their development. In each case, withholding phosphorus from the plants resulted in an increase in the activity of the *de novo* arginine biosynthetic pathway, which paralleled the accumulation of arginine in the leaves of phosphorus-deficient plants (Table 2).

TABLE 1

NANOMOLES $NaH^{14}CO_3$ INCORPORATED INTO ARGININE AND UREA BY INTACT ROOTS EXCISED FROM 2-DAY-OLD SQUASH SEEDLINGS[a]

Addition	Arginine	Urea
None—control	20 ± 0.6	11 ± 3
+L-Ornithine (5 mM)	467 ± 42	25 ± 4
+NH_4Cl (50 mM)	32 ± 6	125 ± 8
+L-Ornithine (5 mM) and + NH_4Cl (50 mM)	232 ± 32	86 ± 13

[a] Data are the average results of three separate experiments ± SE.

TABLE 2

ARGININE CONTENT AND ACTIVITY OF THE ARGININE BIOSYNTHETIC PATHWAY IN LEAVES FROM PHOSPHORUS-SUFFICIENT AND PHOSPHORUS-DEFICIENT CITRUS ROOTSTOCK CULTIVARS

Cultivar	Treatment	μmol arginine per gram dry wt[a]	−P:+P	nmol $NaH^{14}CO_3$ incorporated into arginine and urea per gram fresh wt · 3 h[b]	−P:+P
Rough lemon	−P	207	2.9	91	2.7
	+P	71		34	
Carrizo citrange	−P	308	4.9	49	4.6
	+P	62		11	
Troyer citrange	−P	241	3.7	35	3.2
	+P	65		11	
Trifoliate orange	−P	178	2.0	52	1.3
	+P	88		41	

[a] Each value represents the mean from two separate experiments. The difference between any two values did not exceed 30%.

[b] Values are the average of two separate experiments. The difference between any two replicates did not exceed 20%.

DISCUSSION

The disadvantages of conventional chromatographic procedures for the isolation of radiolabeled metabolites have been reviewed recently (6). Isolation of metabolites by co-crystallization with carrier (6) has provided an alternative method for successfully isolating specific radiolabeled metabolites, free from contaminants, from the significant number of radiolabeled compounds synthesized when $NaH^{14}CO_3$, or other simple radiolabeled precursors, is employed to measure the activity of a biosynthetic pathway in intact cells or tissues. We have had considerable success in applying this technique to plant cells (5,10). However, the low activity of the *de novo* arginine pathway in vascular plants, the fact that arginine, not urea, accumulated in most of the physiological states we examined, and our desire to use $NaH^{14}CO_3$ as the precursor to permit simultaneous determination of its incorporation into orotic acid or ΣUMP necessitated the development of a method more sensitive than existing coprecipitation or chromatographic procedures. Application of commercial enzymes to quantify the amount of $NaH^{14}CO_3$ incorporated into arginine and/or urea provided the needed sensitivity and permitted the study of the regulation of the *de novo* arginine and pyrimidine nucleotide biosynthetic pathways (11).

A potential problem unique to methods employing exogenously supplied enzymes to measure the activity of a pathway in cells of different physiological states is that the change in physiological state is often associated with, or effected by, changes in the level of endogenous or exogenous supplies of metabolites, one or more of which may influence the activity of the commercial enzyme. Consideration of this potential problem dictates that recovery of [*guanido*-^{14}C]arginine from the reaction mixture in question be tested. Thus far, we have not encountered this problem, and the procedure has proven reliable, inexpensive, and relatively rapid; results are obtained within 2 working days.

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