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Properties and Subcellular Distribution of Two Partially Purified Ornithine Transcarbamoylases in Cell Suspensions of Sugarcane[1]

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ABSTRACT

Two spatially separated forms of ornithine transcarbamoylase (EC 2.1.3.3) of different molecular weights coexist in sugarcane (*Saccharum* sp.). The smaller form of the enzyme (mol wt 79,000) appears to be cytoplasmic, while a larger form (mol wt 224,000) sedimented with mitochondria. The K*m* of the cytoplasmic enzyme for ornithine was 3.11 mM, while the enzyme in the mitochondrial fraction had a K*m* of 0.50 mM for this substrate; both enzymes had similar affinity for carbamoyl phosphate (0.12 mM). Characteristics of the smaller ornithine transcarbamoylase are in keeping with a predominantly catabolic function, those of the enzyme which sediments with mitochondria, with an anabolic function. Only the mitochondrial enzyme was regulated *in vivo* by exogenous arginine.

Ornithine transcarbamoylase (EC 2.1.3.3) (OTC)[2] is of physiological importance for the carbamoylation of ornithine, as well as for the decarbamoylation of citrulline. The first reaction is significant because of its role in the arginine biosynthetic pathway, while for some organisms the reverse reaction has a function in the arginine dihydrolase pathway. In mammals (4) and fungi (24), OTC is located exclusively in the mitochondria, while in plants, OTCs of cytoplasmic (6) as well as mitochondrial (2) origin have been reported. Two forms of the enzyme were distinguished in *Pisum sativum* (3) and appear to exist also in apple leaf tissue (20), but their intracellular locations were not determined. On the other hand, Patil (private communication) has found OTC activity not only in the mitochondria and cytoplasm of *Phaseolus vulgaris*, but also in chloroplasts.

Multiple OTCs in bacteria represent separate anabolic and catabolic forms under separate regulatory control with respect to arginine (21). Here we present evidence that two OTCs exist in sugarcane cell suspensions. They differ in their subcellular locations and in many of their physical and kinetic properties. Cell levels of the two forms also respond differently to added arginine.

MATERIALS AND METHODS

Cell Culturing. Unless otherwise noted, sugarcane cells (Saccharum sp.), variety H50-7209) in suspension culture were grown in a stock medium containing yeast extract and supplemented with 300 μM arginine (11). Cells from the stationary phase of growth were harvested and washed.

Enrichment of Mitochondria. One to 5 g fresh weight of cells were washed in 2% sucrose and were ground (mortar and pestle) in the cold, using a high ionic strength buffer (G. M. Richards, private communication) consisting of 0.5 M sucrose, 0.5 M K-phosphate, 0.42 M tris, 1 mM EDTA (sodium salt), and 0.075% BSA (pH 7.2). The initial grinding was done in 1 ml of the buffer, and the resulting homogenate was diluted 1:10 with a buffer containing 0.3 M sucrose, 0.1 M K-phosphate, and 0.08 M tris (pH 7.2) before filtering the homogenate through cheesecloth. The filtrate was centrifuged for 5 min at 250*g*, and the pellet was discarded. The supernatant was centrifuged for 20 min at 10,000*g* to obtain the mitochondrial pellet and postmitochondrial, soluble fraction.

Extraction of Enzymes. The postmitochondrial, soluble fraction was brought to 60% (w/v) with solid $(NH_4)_2SO_4$; protein precipitate was dissolved in 0.01 M tris (pH 8) and dialyzed overnight against the same buffer. This preparation was designated as OTC_C.

Mitochondria in the pellet were washed with extracting buffer and resuspended in 0.01 M tris (pH 8) for 1 hr. During this period the mitochondria were permitted to break, and debris was subsequently removed by centrifugation (15,000*g* for 5 min). Protein in the supernatant was precipitated, resolubilized, and dialyzed as described for the soluble fraction. This preparation was designated as OTC_M.

Determination of OTC. In the direction of citrulline synthesis, OTC was assayed essentially by the method of Ong and Jackson (12), using 1 ml of 0.1 M tris (pH 8), 5 mM ornithine, and freshly prepared 5 mM carbamoyl-P. Enzyme preparations were added to produce between 30 and 100 nmol of citrulline during a 10-min incubation period at 37 C. Citrulline was determined according to Prescott and Jones, method II (13).

OTC in the direction of ornithine formation was assayed by the radioisotope procedure of Reichard (14) which measures $^{14}CO_2$ released from ^{14}C-ureido-labeled citrulline (New England Nuclear) in the presence of arsenate and enzyme. Unlabeled citrulline was added as shown under "Results," and 0.5 ml of extract was used with 1 ml of the reaction mixture, containing 0.1 M citrate buffer (pH 7) and 50 mM arsenate. Incubations in side armed flasks were for 1 hr at 30 C, and the reaction was terminated by addition of 0.5 ml of 6 N HCl and 15% trichloroacetic acid from the side arm. Radioactivity was measured in a liquid scintillation counter (model LS-150, Beckman Instruments Inc., Palo Alto, Calif.) in scintillation fluid containing PPO, POPOP, and toluene. TLC of the assay mixture after incubation showed unreacted citrulline as the only radioactive substance.

Determination of Succinic Dehydrogenase (SDH). SDH was assayed according to the method described by Veeger *et al.* (22).

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[2] Abbreviations: OTC: ornithine transcarbamoylase; SDH: succinic dehydrogenase; GPI: glucose-6-phosphate isomerase.

Controls were included to correct for interfering oxidase reactions in cytoplasmic extracts. No depression of SDH activity by cytoplasmic extracts was observed.

Determination of Glucose-6-phosphate Isomerase (GPI). To eliminate sucrose interference in the assay procedure for GPI, 0.5 M mannitol instead of sucrose was used in the extraction medium for mitochondria. GPI was assayed in mitochondrial and postmitochondrial extracts by a combination of the procedures of Reithel (16).

Molecular Weight Estimation. OTC_C and OTC_M were chromatographed separately on a Sephadex G-200 column (2.5 × 44.5 cm) at a flow rate of 0.1 ml/min; V_c = 213 ml, V_o = 55 ml. The column was calibrated with proteins of known mol wt (peroxidase, alkaline phosphatase, β-amylase, and urease) which were identified by relative peak heights (A_{280}) and by enzymic activity.

Protein Determination. Protein was measured by the method of Lowry *et al.* (7).

RESULTS

Intracellular Distribution of OTC. In one preparation, total OTC activity in the postmitochondrial, soluble fraction was 10 times greater than in the mitochondrial fraction (Table I), but, expressed in terms of protein, the enzyme designated as OTC_M had greater specific activity. Comparison with SDH indicated that OTC_M was associated with a fraction that also contained most of the mitochondria. The distributions of OTC_C and OTC_M were compared with GPI as well as SDH by per cent distribution. Contamination of the postmitochondrial fraction by broken mitochondria averaged 27%, as shown by SDH activity (four separate extractions). However, postmitochondrial contamination of mitochondria was very low, since only 4% of GPI activity occurred in the mitochondrial fraction (three separate extractions). Total OTC activity was 75% in the post mitochondrial fraction and 25% in the mitochondria (12 separate extractions), and this distribution is significantly different from either marker enzyme at 95% confidence level by Mann-Whitney U-test (8).

Subcellular distribution of OTCs was investigated in extracts from the mitochondrial pellet and from the postmitochondrial fraction of sugarcane cells. OTCs in these extracts had different retention characteristics on a DEAE-cellulose column (Fig. 1). OTC_M from the principal peak of the mitochondrial fraction eluted when a concentration of approximately 0.47 M phosphate was reached, whereas OTC_C eluted with a much lower phosphate concentration (0.15 M).

Kinetic Properties. In the forward reaction, and with an ornithine concentration of 10 mM, OTC_C had a sharp peak of activity at pH 7.5, while OTC_M activity had a broader optimum pH,

Table I. Distribution of Ornithine Transcarbamoylase Activity in Subcellular Fractions

	Mitochondrial fraction	Post-mitochondrial fraction
Ornithine transcarbamoylase		
Specific activity[1]	100.0	40.2
Total activity[2]	12.0	126.6
Succinic dehydrogenase		
Specific activity[3]	6.00	0.06
Total activity[4]	0.72	0.20
Total protein (mg)	0.12	3.15

[1] nmol citrulline formed/min • mg protein
[2] nmol citrulline/min • g fresh wt cells
[3] Change in A_{600}/min • mg protein
[4] Change in A_{600}/min • g fresh wt cells

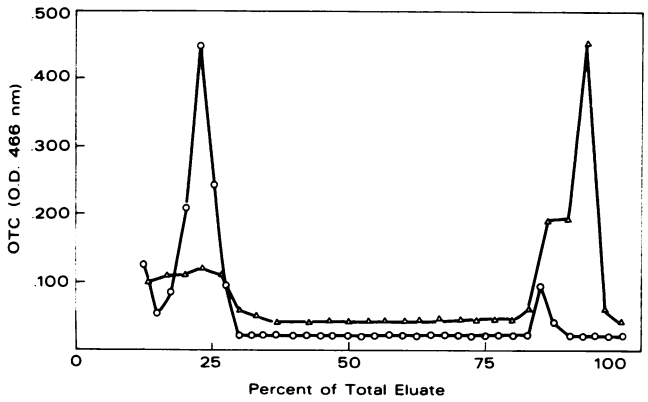

FIG. 1. Elution of OTC_C (O——O) and OTC_M (Δ——Δ) on DEAE-cellulose. Enzymes were eluted by a linear gradient of 0.05 to 0.5 M K-phosphate buffer (pH 7.5) from a column (2 × 15 cm) at a flow rate of 0.3 ml/min. Volumes in the mixing flasks were 400 ml for OTC_C and 200 ml for OTC_M.

Table II. Comparison of the Effect of pH on OTC Activity in the Forward and Reverse Reactions

Conditions used for the assays were those described under Materials and Methods, except that the ornithine concentration for the forward reaction was 10 mM. Redetermination of pH values after incubation showed that they remained constant within ±0.10 pH units.

	Citrulline formation		Ornithine formation	
pH	OTC_M	OTC_C	OTC_M	OTC_C
	%		%	
6.0	--	--	35	1
6.5	--	--	29	26[4]
7.0	28	55	100[3]	100[4]
7.5	92	100[2]	36	15
8.0	100[1]	57	0	0
8.5	91	30	--	--
9.0	78	16	--	--

[1] 46.8 nmol/min • mg protein
[2] 21.9 nmol/min • mg protein
[3] 1.23 nmol/min • mg protein
[4] 0.89 nmol/min • mg protein

ranging from pH 7.5 to 8.5 (Table II). In the reverse reaction, the activity of both enzymes peaked sharply at pH 7.

The effect of pH on the forward reaction (citrulline formation) depended on ornithine concentrations in the incubation mixture. When pH was kept constant, there was appreciable substrate inhibition, particularly of OTC_C (Fig. 2). At pH 8.5, maximum activity for both OTC_C and OTC_M was reached at about 1 mM ornithine. At higher ornithine concentrations, there was a rapid decrease in activity. At pH 8, the pattern for OTC_C was similar, although substrate inhibition was less severe. At this pH, OTC_M had a much broader activity spectrum: maximum activity occurred between 3 and 4 mM ornithine, and inhibition by ornithine was only 23%, even at a substrate concentration of 10 mM.

When ornithine was added at concentrations below that needed to produce inhibition, the activity of both OTCs increased up to pH 9, the highest pH tested (Table III). On the other hand when ornithine concentrations were adjusted to yield a constant concentration of the zwitterion form (pK_a 8.65) at

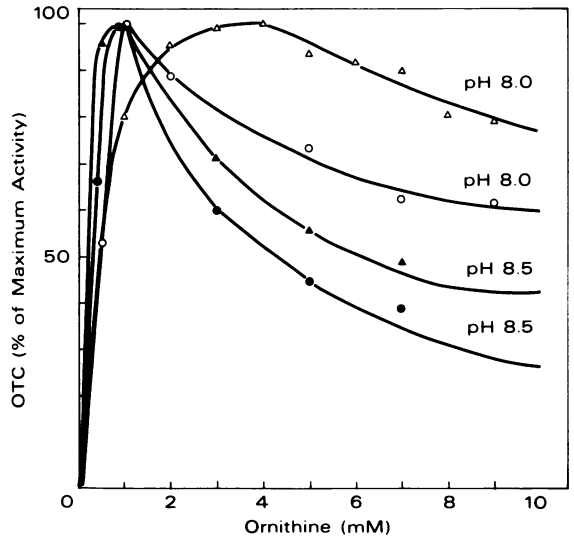

FIG. 2. Effect of ornithine concentration on OTC_C (○——○, and ●——●) and OTC_M (△——△, ▲——▲) in the direction of citrulline formation at pH 8 (○——○, △——△) and pH 8.5 (●——●, ▲——▲). Control rate (100%) for OTC_C was 33 nmol product/min·mg protein and for OTC_M 64 nmol/min·mg protein.

Table III. Effect of pH on OTC's at Constant Ornithine Concentration (0.5 mM) in the Direction of Citrulline Synthesis

Activities are expressed as % of activity at pH 9.0.

pH	OTC_C	OTC_M
	%	
7.0	15	12
7.5	26	31
8.0	52	63
8.5	73	93
9.0	100[1]	100[2]

[1] 45 nmol/min • g fresh wt cells

[2] 3.2 nmol/min • g fresh wt cells

Table IV. Effect of pH on OTC Activities at Non-Saturating Ornithine Concentrations

Ornithine concentrations were varied to give equal concentrations of α-NH_2 species (pK 8.65) at each pH value. Percentage activities are based on activities of OTC's at optimal ornithine concentrations (2.0 mM for OTC_C[1], and 3.0 mM for OTC_M[2]) at pH 8.0.

pH	Orn	OTC_C	OTC_M
	mM	%	
7.5	1.40	41	67
8.0	0.50	46	65
8.5	0.22	64	65
9.0	0.13	50	63

[1] 1.8 O.D. units/10 min • 0.1 ml extract

[2] 0.643 O.D. units/10 min • 0.1 ml extract

each pH, OTC activity remained relatively constant (Table IV).

At pH 8 and a saturating concentration of carbamoyl-P, Michaelis-Menten kinetics prevailed for OTC_C and a *Km* value of 3.11 mM was calculated for ornithine (Fig. 3A). OTC_M had a much higher affinity for ornithine (*Km* = 0.5 mM) (Fig. 3B). The Lineweaver-Burk plots indicated that high ornithine concentrations inhibit OTC_C more than OTC_M. No inhibition was observed when *Km* values for carbamoyl-P were determined in a similar manner and no appreciable differences of the *Km* values for carbamoyl-P were found between the two enzymes (OTC_C = 0.12 mM; OTC_M = 0.11 mM) (plots not shown).

Contrary to findings for the forward reaction, the formation of ornithine from citrulline required not only a higher concentration of substrate for saturation, but OTC_C had a higher affinity for citrulline than did OTC_M (Fig. 4A and B).

Relative Molecular Size. An approximation of mol wt of the two OTCs was obtained on Sephadex G-200 by comparing their displacement from a column with that of several enzymes of known mol wt. On this basis mol wt of OTC_C and OTC_M were calculated as 79,000 and 224,000, respectively (data not shown).

Inhibition by Arginine. Previously (9), it was demonstrated that cultures of aging cells grown in the absence of exogenous arginine divide less rapidly than similar cells grown in media containing arginine. We compared the specific activities of the two OTCs in cells grown for 6 to 8 days in the presence and absence of exogenous arginine (Table V). The activity of OTC_M increased between 2- and 3-fold in the absence of arginine, while OTC_C did not respond to arginine supplementation; its activity, under both conditions, remained comparable to that of OTC_M in the arginine-supplemented medium. Results were variable but statistically significant.

In vitro, arginine did not inhibit OTC_M or OTC_C, even when arginine exceeded the maximum concentration at which it is likely to be present in the cells by a factor of 10 (18).

DISCUSSION

Existence of two OTCs in surgarcane cell suspensions corroborates reports by others that higher plants have multiple forms of this enzyme (3, 20). The two OTC forms differ in their relative mobilities on gel filtration, their intracellular locations, and their kinetic properties with respect to ornithine and citrulline. Cell levels of OTC_M but not OTC_C are decreased in the presence of exogenous arginine. The two forms are similar in their affinities for carbamoyl-P in the forward reaction. The elution pattern of sugarcane OTCs from a DEAE-cellulose column resembled the elution pattern from a similar column reported for pea seedlings (3). Multiple OTCs from pea seedlings (3) and apple leaf tissue (20) have been distinguished on the basis of their pH optima in the forward reaction. However, in sugarcane, OTCs do not differ fundamentally in their pH response. For both forms, as for human liver OTC, pH appears to affect activity largely by the concentration of the ornithine zwitterions present and these may be the true substrate at both catalytic and inhibitor-binding sites (19). Differences in pH optima are related to differences in *Km* and *Ki* values for ornithine and are dependent on the concentration of ornithine in the incubation mixture.

Mol wt calculated from a molecular sieve column are approximations, but OTC_M theoretically could be a trimer of the molecular form of the cytoplasmic sugarcane enzyme. Similar mol wt relationships exist in OTCs of *Streptococcus faecalis* and bovine liver (10), enzymes normally having stable species of 223,000 and 108,000, respectively. In 6 N guanidine HCl, both of these enzymes form a monomeric species of approximately 38,000 mol wt, and the *S. faecalis* enzyme forms a stable dimer (*i.e.* 74,000 mol wt) at pH 9.5 in 0.2 M NH_4Cl, a species similar to sugarcane OTC_C, as well as to *Nostoc muscorum* OTC (1). Marshall and Cohen (10) propose that the enzyme is further

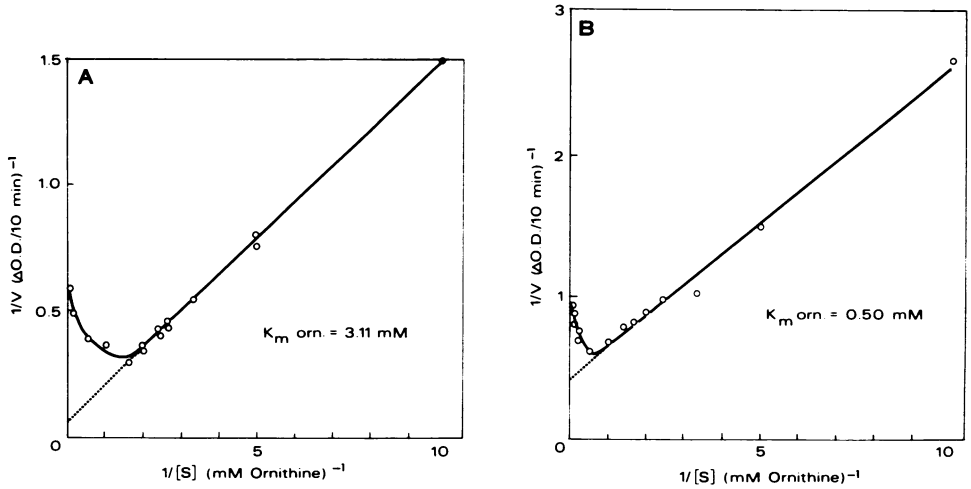

FIG. 3. Double reciprocal plots showing saturation of OTC_C (A) and OTC_M (B) by ornithine. carbamoyl-P concentration was 5 mM and the pH was 8. Lines were fitted by the least squares method.

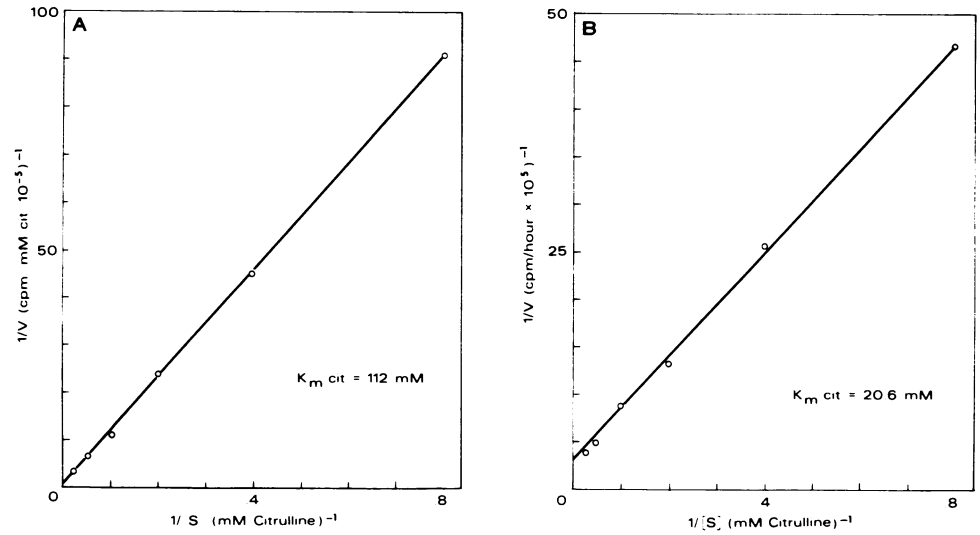

FIG. 4. Double reciprocal plots of OTC_M (A) and OTC_C (B) functioning in the reverse direction, pH 7, using citrulline as a substrate.

stabilized by a hexamer configuration (*i.e.* 224,000 mol wt) of the basic unit, but that the dimer also has functional significance.

Intracellular location of OTCs in plants is not known, but our data suggest that the high mol wt OTC_M, isolated by a method yielding chiefly mitochondria, is, in fact, a mitochondrial enzyme. The smaller enzyme, OTC_C, obtained from the supernatant of a 10,000*g* centrifugation is likely to be cytoplasmic.

Functionally, the two OTCs may differ. The enzyme in the particulate fraction appears to favor an anabolic reaction, while kinetics for the soluble OTC indicates a catabolic role for this enzyme. A physical separation of two functionally different OTCs offers advantages in economizing cell energy (23) and in metabolic regulation (25).

In the direction of ornithine synthesis, the affinity of OTC_C for citrulline is higher than affinity of OTC_M for this substrate. In the direction of citrulline formation, on the other hand, the situation is reversed: OTC_M has a higher affinity for ornithine than does OTC_C. Further evidence for the relative inefficiency of OTC_C as an anabolic enzyme is substrate (ornithine) inhibition which begins to limit activity well before the theoretical *Km* for ornithine is attained, whereas OTC_M is inhibited by ornithine only when the saturation limit of the enzyme-substrate complex is approached. The relationship between mol wt of *Pseudomonas* OTCs and their respective metabolic functions differ from similar relationships for sugarcane cell OTCs: in *Pseudomonas* it is the smaller OTC that is associated with an anabolic function, and the larger which has a catabolic function (25); in sugarcane we find the reverse situation.

OTCs in microbial species are under different regulatory controls with respect to arginine (17, 25). In sugarcane cells, OTC_M, the anabolic enzyme, is least abundant when the cells are grown in a medium containing arginine. Therefore, it resembles the anabolic enzyme in *Pseudomonas* which also has a similar pH optimum at 8 to 8.5 (21). OTC with an anabolic function (*i.e.* OTC_M) is more likely to be subject to repression by exogenous arginine than is the catabolic OTC (*i.e.* OTC_C), but, unlike argininosuccinate synthetase in soybean cells (18), OTC_C is probably not inhibited by an allosteric mechanism because exogenous arginine affects only cells incubated in the presence of arginine for several days. An anabolic function for OTC_M is in keeping with the mitochondrial location of OTC in fungi (24) and mammals (4) and also with observations that isolated mung bean mitochondria converted ^{14}C-ornithine to ^{14}C-citrulline (2). A catabolic function for OTC_C is at present only speculative. Many plants alternately store and release nitrogen from the arginine molecule (6, 15) but catabolism is thought to proceed through arginase and urease rather than the dihydrolase pathway. An examination of the enzymic pathways of arginine catab-

Table V. Effect of Exogenous Arginine on Cytoplasmic and Mitochondrial OTC's in Sugarcane Cells

Flasks were inoculated at start of experiments with 1-week-old washed stock cells from a single culture. OTC in the direction of citrulline synthesis was determined after 6 to 8 days' incubation of the cells in a synthetic medium (11) supplemented with 300 μM L-arginine (+), or without arginine (-).

Experiment	OTC_C		OTC_M	
	+	-	+	-
	nmol/min • mg soluble or mitochondrial protein			
1	54	28	44	157
2	166	186	134	220
3	44	44	43	86
4	53	35	38	146
Mean	79	73	65	152
$S_{\bar{D}}$	10.4		16.1	
t^{D}	0.58		5.47[1]	

[1] Significant difference between means at 95% confidence interval.

olism in sugarcane cells is now important, not only to determine the function of OTC_C but also because the dihydrolase pathway, in contrast to the arginase pathway, yields ATP from the decarbamoylation of citrulline (5), and could contribute significantly to the energy budget of plant tissues in heterotrophic stages of growth.

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