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BIOSYNTHESIS OF N²-(1,3-DICARBOXYPROPYL) ORNITHINE [NOPALINIC ACID] IN CROWN GALL TUMOUR TISSUE

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco, *Agrobacterium tumefaciens*; crown gall tumours; biosynthesis; amino acid derivatives; N^2-(1,3-dicarboxypropyl)ornithine; nopalinic acid.

Abstract—Protein extract from crown gall tumour tissue, induced on *Nicotiana tabacum* by *Agrobacterium tumefaciens* strain T37, synthesized nopalinic acid [N^2-(1,3-dicarboxypropyl)ornithine] from L-ornithine and α-ketoglutarate in the presence of NADPH. Label was incorporated into nopalinic acid from both L-ornithine-[^{14}C] and α-ketoglutarate-[^{14}C] *in vivo*. Nopaline [N^2-(1,3-dicarboxypropyl)arginine] did not appear to be metabolized to nopalinic acid *in vivo*.

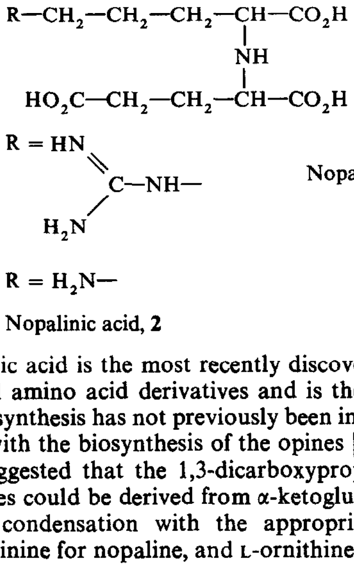
INTRODUCTION

Agrobacterium tumefaciens is a tumour-inducing plant pathogenic bacterium with a wide host range [1]. The oncogenic virulence of *A. tumefaciens* is associated with a large plasmid [2] and there is evidence that a portion of this Ti plasmid is incorporated into the plant cell [3] and therein transcribed [4]. 'Crown gall' tumours commonly contain unusual amino acid derivatives of two types, depending on the type of Ti plasmid present in the initiating bacterium [5–8]. Firstly the 'opine' family of N^2-(1,3-carboxyethyl) derivatives of L-arginine (octopine) [9], L-ornithine (octopinic acid) [10], L-lysine (lysopine) [11, 12] and L-histidine (histopine) [13]; secondly the 'aline' family of N^2-(1,3-dicarboxypropyl) derivatives of L-arginine (nopaline, **1**) [14] and L-ornithine (nopalinic acid, **2**) [15]. There has been no convincing demonstration of the presence of these compounds in normal plant tissue [16, 17]. Bacterial strains which induce tumours containing opines or alines can specifically metabolize the same family of compounds [5, 13, 15, 16, 18, 19].

Table 1. Requirements for synthesis of nopalinic acid by T37CG protein extract. Results are expressed as relative synthesis of nopalinic acid-[^{14}C] for each set of assays

Reaction mixture	Relative synthesis
(a) Synthesis from L-ornithine-[^{14}C]	
Complete	100
minus α-ketoglutarate	5.5
minus NADPH	3.2
(b) Synthesis from α-ketoglutarate-[^{14}C]	
Complete	100
minus L-ornithine	8.8
minus NADPH	4.7
(c) Synthesis from L-ornithine-[^{14}C]	
NADPH as cofactor	100
NADH as cofactor	20
without cofactor	<5.8

Each value is a mean of duplicates.



Nopaline, **1**

Nopalinic acid, **2**

Nopalinic acid is the most recently discovered of the crown gall amino acid derivatives and is the only one whose biosynthesis has not previously been investigated. Analogy with the biosynthesis of the opines [10, 13, 16, 20–25] suggested that the 1,3-dicarboxypropyl moiety of the alines could be derived from α-ketoglutarate by a reductive condensation with the appropriate amino acid: L-arginine for nopaline, and L-ornithine for nopalinic acid. There is evidence for this in the case of nopaline [20, 21]. The present paper describes *in vitro* and *in vivo* studies on the biosynthesis of nopalinic acid.

Table 2. Amino acid metabolites of α-ketoglutarate-[^{14}C] in crown gall tumour tissue. Results are given for an extended 'glutamate–ornithine' family of amino acids most likely to be metabolically related to nopaline and nopalinic acid

Compound	^{14}C present in each compound (nCi)	
	T37CG	B6CG
Nopaline	14.1	0.2
Nopalinic acid	8.6	0.2
Glutamate	4.4	7.6
Glutamine	11.7	0.9
Ornithine	0.2	0.7
Arginine	0.7	0.4
γ-aminobutyrate	8.5	66.6
Proline	0.2	0.1

Each value is a mean of duplicates.

Table 3. Distribution of ^{14}C between amino acid metabolites of L-ornithine-[^{14}C] in T37CG tissue, and effect of co-feeding unlabelled α-ketoglutarate

Compound	(a) L-ornithine-[^{14}C] (%)	(b) L-ornithine-[^{14}C] + 500 μg unlabelled α-ketoglutarate (%)
Nopaline	54.3	15.1
Nopalinic acid	40.0	20.4
Glutamate	0.3	0.7
Glutamine	1.0	1.3
Ornithine	2.9	57.8
Arginine	0.6	1.0
γ-aminobutyrate	1.0	1.9
Proline	0.1	1.9

Each value is a mean of duplicates.

RESULTS

A protein extract of tissue culture line T37CG incubated with L-ornithine-[^{14}C], α-ketoglutarate and NADPH synthesized a radioactive product which co-chromatographed with authentic nopalinic acid (2D PC, solvents *A/C*, *A/D*, see Experimental). GC–MS of the heptafluorobutyric (HFB) *n*-propyl derivative [26] of this product gave an identical spectrum to that of nopalinic acid (32.1 % conversion). Protein extract from B6CG (an opine-containing tumour line) was inactive (<0.25 % conversion) (although it was active in glutamate-α-ketoglutarate transaminase), as was boiled T37CG protein extract (<1.5 % conversion). T37CG protein extract also synthesized nopalinic acid-[^{14}C] from α-ketoglutarate-[^{14}C] and L-ornithine, and the reaction showed dependence on the presence of both substrates and NADPH, which was preferred to NADH as cofactor (Table 1).

In vivo feeding experiments with T37CG tissue confirmed that α-ketoglutarate was a precursor of nopalinic acid (and also nopaline). B6CG tissue did not synthesize either compound (Table 2). However, L-ornithine-[^{14}C]-feeding did not specifically label nopalinic acid in T37CG tissue; nopaline was also labelled (Table 3a), suggesting that metabolism of L-ornithine-[^{14}C] to α-ketoglutarate-[^{14}C] may have occurred. Co-feeding unlabelled α-ketoglutarate with L-ornithine-[^{14}C] diminished the labelling of the alines relative to L-ornithine (Table 3b), but this could have been due either to dilution of an α-ketoglutarate-[^{14}C] pool or to dilution of the L-ornithine-[^{14}C] pool by conversion of the unlabelled α-ketoglutarate to L-ornithine.

Another possible biosynthetic derivation of nopalinic acid might be the hydrolysis of nopaline by an enzyme analogous to arginase. However, when L-arginine-[^{14}C] was fed to T37CG tissue, over 90 % of the ^{14}C in the free amino acid pool was found in nopaline, without significant labelling of nopalinic acid 24 hr or 1 week after feeding, and co-feeding unlabelled nopalinic acid did not cause any accumulation of label in the nopalinic acid pool (Table 4a). Likewise, nopaline-[^{14}C]-feeding did not cause any accumulation of label in the nopalinic acid pool (Table 4b).

Table 4. (a) Relative incorporation of label from L-arginine-[^{14}C] into nopaline and nopalinic acid in T37CG tissue. (i) 24 hr incubation; (ii) 1 week incubation; (iii) as (i), but with co-feeding of unlabelled nopalinic acid in *ca* 10-fold excess over tissue content. Results are expressed as % of total ^{14}C detected in the amino acids listed in Table 2.

Compound	Incorporation of ^{14}C from L-arginine-[^{14}C]		
	(i)	(ii)	(iii)
Nopaline	96.7 %	95.2 %	93.9 %
Nopalinic acid	0.8	0.6	1.1
Arginine	1.7	2.4	3.3

(b) Distribution of label in T37CG tissue after feeding nopaline-[^{14}C]

Compound	^{14}C present in each compound (nCi)
Nopaline	158.9
Nopalinic acid	0.8
Arginine	0.4

Each value is a mean of duplicates.

DISCUSSION

The *in vitro* evidence suggests that nopalinic acid is synthesized in crown gall tumour tissue from L-ornithine and α-ketoglutarate by an NADPH-dependent enzyme. Confirmation of this reaction must await the purification of the enzyme. Comparable biosyntheses have been demonstrated for the other amino acid derivatives [16, 21–24]. Nopalinic acid and nopaline may be synthesized by the same enzyme, as Hack and Kemp [23] suggest for the 4 opines.

Unequivocal evidence for the biosynthetic reaction demonstrated *in vitro* could not be obtained *in vivo* because of the close metabolic relationship of the two postulated precursors.

An *in vivo* conversion of nopaline to nopalinic acid could not be demonstrated. This parallels the situation in the opine family, since octopinic acid does not appear to be formed from octopine [10, 20].

It is unlikely that the amino acid derivatives are essential for the tumorous nature of crown gall tissue since certain tumours do not contain them at detectable levels [5, 7]. However, large quantities are synthesized by some tissues (in T37CG, *ca* 20 mg/g dry wt nopaline and *ca* 15 mg/g nopalinic acid; Butcher *et al.* unpublished), and could represent a conversion of the plant's nutritional resources into a form which can be utilized specifically by appropriate strains of *A. tumefaciens* [7].

EXPERIMENTAL

Crown gall tumours. Tissue culture line T37CG, initiated on *Nicotiana tabacum* with *A. tumefaciens* strain T37, was obtained from Dr D. N. Butcher, Rothamsted Experimental Station. It was cultured on the fortified salts medium of ref. [27] at 20 in diffuse light. Tumour line B6CG was initiated by strain B6 on *N. tabacum* cv White Burley and cultured as described in ref. [15]. Both culture lines were bacteria-free.

Chemicals. Nopaline [28] and nopalinic acid [15] were synthesized chemically. Radiochemicals: 2-keto [5-^{14}C] glutaric acid Na salt, 10.3 m Ci/mmol; L-ornithine [1-^{14}C] HCl, 60 mCi/mmol, L-arginine-[U-^{14}C] HCl, 324 mCi/mmol.

Chromatographic methods. Descending chromatograms were run on Whatman 3 MM paper. Solvent systems: *A*, *n*-PrOH–0.88 NH_4OH (3:2); *B*, PhOH–EtOH–H_2O–0.88 NH_4OH (120:40:40:1); *C*, Py-*iso*-AmylOH–H_2O (8:4:7); *D*, *sec*-BuOH–HCO_2H–H_2O (15:3:2). Amino acids were located by dipping the paper through 0.25% ninhydrin in Me_2CO and heating at 60°. Nopaline was located by the Sakaguchi reaction: papers were dipped through 0.1% 8-hydroxyquinoline in Me_2CO and then sprayed with 0.2% (v/v) Br_2 in 0·5 M aq. NaOH. Labelled compounds were located by autoradiography or by comparison with internal standards. Quantitative measurement of ^{14}C was by liquid scintillation spectrometry. All counts were corrected for counting efficiency and quenching.

Radioisotope feeding expts. Tumour tissue (100–200 mg fr. wt) was injected with the labelled substrate in aq. soln (10–40 μl) and incubated in the dark at *ca* 20° for 15–25 hr. The tissue was then frozen in liquid N_2 and freeze-dried. The dry tissue was ground with acid-washed sand and twice extracted with 20 ml H_2O for 1 hr at 4°. Amino acids were isolated from the extract supernatant by cation exchange (Zerolit 225, 52–100 mesh, H^+ form, 5 ml bed vol.; elution with 50 ml 2M NH_4OH, which was evapd at 45°), and analyzed by 2D PC (solvents *A*/*B*).

Isolation of nopaline-[^{14}C] *for feeding expts.* T37CG tissue was incubated with L-arginine-[^{14}C] (3 μCi), and nopaline-[^{14}C] isolated from the extracted amino acids by prep 2D PC (solvents *A*/*C*). (This procedure did not yield any residues inhibitory to metabolism of L-arginine-[^{14}C] in T37CG tissue.) The extracted nopaline was assayed colorimetrically by the diacetyl/α-naphthol method [29]. Radiochemical yield *ca* 20%; purity *ca* 90% (PC, solvent *A*). Mean sp. act. (duplicate prepns) 0.9 mCi/mmol.

Protein extraction. Fresh tumour tissue was homogenized in Tris–HCl buffer (0.1 M, pH 8) containing 0.5 M sucrose, 0.1% ascorbic acid and 0.1% cysteine HCl. After cheesecloth-filtration, the homogenate was centrifuged at 5000 *g* (10 min) and the supernatant re-centrifuged at 70000 *g* (90 min). The final supernatant was applied to a Sephadex G-50 column pre-equilibrated with Tris–HCl buffer (0.05 M, pH 8) containing 2×10^{-4} M dithioerythritol (DTE). After elution with the same buffer, the protein fraction was pptd with $(NH_4)_2SO_4$ (50% w/v) for 1 hr. After centrifugation (25000 *g*, 30 min), the protein pellet was re-suspended in pH 7.5 buffer (40 mM K_2HPO_4, 12 mM KH_2PO_4) containing 2×10^{-4} M DTE and 5% (v/v) glycerol, and dialyzed for 15 hr against the same buffer. The soln was then cleared by centrifugation (150000 *g*, 4.5 hr). The whole procedure was carried out at 4°. Nopalinic acid-synthesizing activity in the extract was stable for at least one month at −20°.

Enzyme assay. The standard reaction mixture contained protein extract (0.1 ml), L-ornithine HCl (6.7 mM), α-ketoglutarate (6.7 mM), NADPH (5.3 mg/ml), Tris–HCl buffer (260 mM, pH 7.5), DTE (2×10^{-4} M), and either L-ornithine-[^{14}C] (0.2 μCi) or α-ketoglutarate-[^{14}C] (1 μCi), in a final vol. of 0.75 ml. Where indicated, β-NADH was substituted for NADPH at the same conc. After incubation (15–24 hr *ca* 20°), amino acids were isolated by sequential cation exchange (Zerolit 225) and anion exchange (Dowex 1-X8, 20–50 mesh, OH^- form, 5 ml bed vol.; elution with 50 ml 2M HOAc, which was evapd at 45°). The ^{14}C-amino acids were analyzed by PC (Solvent *A*).

GC-MS. GLC of the HFB *n*-propyl derivative of nopalinic acid was carried out by the method of ref. [26]; the MS were obtained at 70 eV.

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