Phytochemistry, Vol 25, No. 1, pp. 107–110, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00

ARGININE DECARBOXYLASE AS THE SOURCE OF PUTRESCINE FOR TOBACCO ALKALOIDS*

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(*Received* 12 *May* 1985)

Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; biosynthesis; alkaloids; nicotine; nornicotine; putrescine; arginine decarboxylase.

Abstract—The putrescine which forms a part of nicotine and other pyrrolidine alkaloids is generally assumed to arise through the action of ornithine decarboxylase (ODC). However, we have previously noted that changes in the activity of arginine decarboxylase (ADC), an alternate source of putrescine, parallel changes in tissue alkaloids, while changes in ODC activity do not. This led us to undertake experiments to permit discrimination between ADC and ODC as enzymatic sources of putrescine destined for alkaloids. Two kinds of evidence presented here support a major role for ADC in the generation of putrescine going into alkaloids: (a) A specific 'suicide inhibitor' of ADC effectively inhibits the biosynthesis of nicotine and nornicotine in tobacco callus, while the analogous inhibitor of ODC is less effective, and (b) the flow of ^{14}C from uniformly labelled arginine into nicotine is much more efficient than that from ornithine.

INTRODUCTION

Considerable evidence indicates that polyamines (PAs) and their biosynthetic enzymes arginine decarboxylase, ADC (EC 4.1.1.19) and ornithine decarboxylase ODC (EC 4.1.1.17) may regulate some aspects of plant development, senescence, and response to stress [1, 2]. PAs may also be metabolized into a variety of secondary plant products [2], through largely unelucidated pathways. Thus, putrescine, formed in the plant via ADC and/or ODC (see Fig. 1), is not only the precursor of the major PAs spermidine and spermine, but also of parts of such important secondary metabolites as alkaloids. The pyrrolidine rings of tobacco alkaloids (nicotine, nornicotine), tropane alkaloids (hyoscyamine, hyoscine, meteloidine), pyrrolizidine alkaloids (retronecine), *Erithroxylum* coca alkaloids (hygrine, cuscohygrine), and probably phenanthroindolizidines (tylophorine) are putrescine derivatives [3, 4]. Putrescine, spermidine, homospermidine and spermine can also conjugate with cinnamic acids or fatty acids [5, 6], and in some cases the conjugates are transformed into more complex alkaloids [7].

We have reported [8] that in tobacco callus grown on high auxin, suboptimal for alkaloid formation, putrescine and spermidine conjugates are the main putrescine derivatives, while in callus grown on or transferred to low auxin, optimal for alkaloid formation, nornicotine and nicotine are the main putrescine derivatives. The results suggested that bound putrescine could act as a pool for alkaloid formation. In addition, we found that changes in ADC but not ODC activity corresponded with altered alkaloid levels [8]. This was surprising, since ODC activity has long been invoked to explain the biosynthesis of nicotine [9] and related alkaloids, and in fact, pyrrolidine alkaloids are usually classified as ornithine-derived compounds [3]. To help decide between the ADC and ODC pathways, we have used two recently developed specific, enzyme-activated, irreversible inhibitors of ADC, (DL-α-difluoromethylarginine; DFMA) [10] and ODC

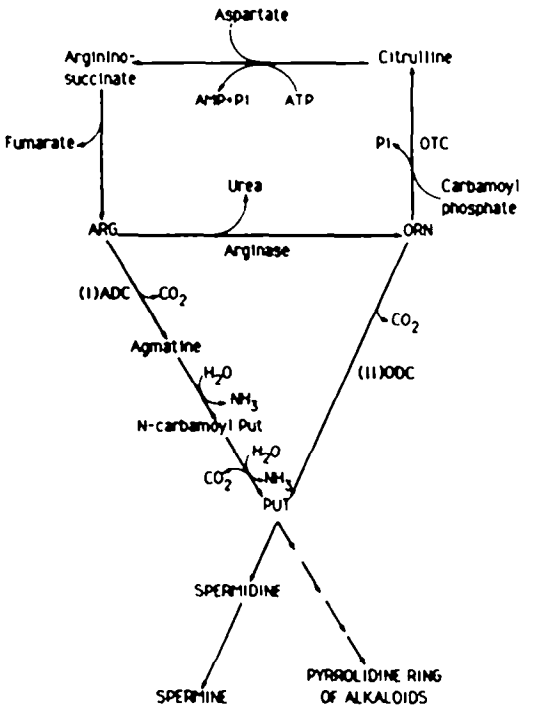

Fig. 1. Schematic pathway for biosynthesis of PA and putrescine-derived alkaloids. Note that only some enzymes are indicated.

*Supported by a grant from the U.S. Israel Binational Agricultural Research and Development Fund (BARD) to AWG.

(DL-α-difluoromethylornithine; DFMO) [11]. We have also employed a reversible inhibitor of ADC, D-arginine (D-Arg), and have supplied the callus with the radiolabelled precursors of putrescine, L-[U-^{14}C]arginine and L-[U-^{14}C]ornithine, to gain additional evidence on paths of carbon flow.

RESULTS AND DISCUSSION

Two different strains of tobacco callus were used in this study. One (NTW-38), derived from *Nicotiana tabacum* 'Wisconsin-38', contains nornicotine as the major alkaloid [12], and a second (NTB-21), derived from *N. tabacum* 'Burley-21' plants, has nicotine as the main alkaloid. Table 1 and Fig. 2 compare the growth of the NTW-38 strain callus in the absence (control) and presence of inhibitors. Addition to the medium of 0.1–3 mM DFMA promoted growth as compared with the control, while DFMO at similar concentrations inhibited callus growth (see also Table 1) and at 10 mM was severely toxic. A combination of the two inhibitors at 1–3 mM (Fig. 2), also produced inhibition of callus growth, apparently due to a predominant action of DFMO. As with DFMA, addition to the medium of 10 mM D-Arg also promoted the growth of NTW-38 strain callus (Table 1).

While these observations suggest that the ODC pathway is important for cell division and growth, the effects of the inhibitors on alkaloid biosynthesis show that ADC is more important in synthesis of pyrrolidine alkaloids. In the NTB-21 callus strain, addition to the medium of 0.1

Table 1. Effect of the inhibitors on fresh weight and alkaloid levels in tobacco callus

	NTW-38 callus strain		NTB-21 callus strain	
Treatment	Fresh weight (g)	Nornicotine (μmol/g dry weight)	Fresh weight (g)	Nicotine (μmol/g dry weight)
Control	2.2 ± 0.04 (100)	85 ± 5 (100)	1.3 ± 0.09 (100)	288 ± 11 (100)
DFMO (0.1 mM)	1.5 ± 0.04 (69)**	67 ± 9 (79)	1.1 ± 0.04 (85)	199 ± 32 (69)
DFMO (1 mM)	1.4 ± 0.14 (62)*	79 ± 14 (93)	0.9 ± 0.09 (69)	167 ± 25 (58)*
DFMO (10 mM)	0.3 ± 0.08 (12)**	N.T.	N.T.	N.T.
DFMA (0.1 mM)	2.5 ± 0.07 (114)	N.T.	1.4 ± 0.03 (108)	173 ± 11 (60)*
DFMA (1 mM)	3.1 ± 0.07 (137)**	N.T.	1.5 ± 0.21 (115)	97 ± 6 (33)**
D-Arg (0.1 mM)	N.T.	N.T.	1.3 ± 0.10 (99)	175 ± 4 (61)*
D-Arg (1 mM)	2.4 ± 0.04 (107)	50 ± 6 (59)*	1.3 ± 0.25 (104)	93 ± 7 (32)**
D-Arg (10 mM)	2.6 ± 0.04 (116)*	44 ± 4 (52)*	N.T.	N.T.

Numbers represent means ± s.e.m. of at least three replicates. Figures in parentheses indicate per cent relative to control. Significantly different from control at the 5% (*) or 1% (**) levels. N.T. = not tested.

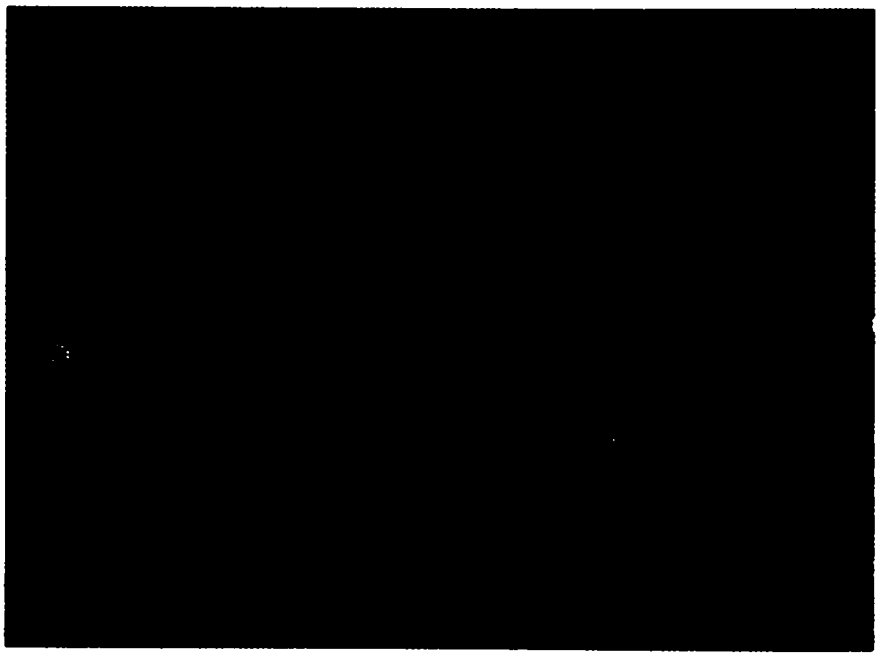

Fig. 2. Effect of the inhibitors alone or in combination on growth of the NTW-38 callus strain.

and 1.0 mM DFMA resulted in calli with respectively, 40% and 60% less nicotine than the control (Table 1). In contrast, 0.1 and 1.0 mM DFMO produced no significant reduction of alkaloid levels in the NTW-38 callus strain; DFMO was also less effective than DFMA in reducing nicotine levels in the NTB-21 callus strain. As with DFMA, addition to the medium of 0.1–10 mM D-Arg was more effective than DFMO in depressing nicotine and nornicotine levels in the two callus strains (Table 1).

To elucidate further the preferential participation of ADC in the biosynthesis of pyrrolidine tobacco alkaloids, NTB-21 calli were fed with ^{14}C-labelled precursors of putrescine. After 6 hr of incubation the calli incorporated 15 and 17% respectively of the initial cpm fed as ^{14}C-arginine and ^{14}C-ornithine, showing therefore similar uptake of the label from the two compounds. However, callus fed with ^{14}C-arginine incorporated more of this label into nicotine than callus fed with ^{14}C-ornithine. As indicated in Table 2, the incorporation ratio for ^{14}C-arginine was about 3-fold higher than for ^{14}C-ornithine and the callus accumulated more nicotine than callus fed with ^{14}C-ornithine.

Interconversions between arginine and ornithine are illustrated in Fig. 1. In tissues with high arginase activity, arginine could be converted into ornithine and DFMA into DFMO, as recently observed [R. D. Slocum, unpublished]. To test this possibility, the arginase levels of the NTB-21 callus strain were analysed. Neither detectable arginase activity nor interference with added bovine liver arginase activity were found in calli.

From the above results, we conclude that in tobacco callus, ODC is important for growth, while ADC appears to be involved in furnishing putrescine for pyrrolidine alkaloid biosynthesis (see Fig. 1, pathway I). This is in agreement with the view [2] that there appears to be a different compartmentation of the two biosynthetic enzymes for putrescine in higher plants. ODC appears to be mainly located in the nuclei and thus may regulate cell division in actively growing tissues, while ADC appears to be located in the cytosol, probably regulating cell extension and secondary processes [R. D. Slocum, unpublished].

The hypothesis that ODC is the main enzyme involved in nicotine biosynthesis is based on equivocal decapitation experiments with tobacco [9, 13]. Following decapitation of the stems, both ODC activity and nicotine levels increased in the roots and these effects were attributed to a decrease of auxin levels in the roots [9]. However, in the roots of non-decapitated plants, ADC was 4.5-fold higher than ODC activity [13], and the roots of these plants also produced alkaloids. Thus, the primary role of ODC in nicotine biosynthesis remains questionable, since effects other than decreased auxin levels in the roots can result from decapitation of the plant. Yoshida [14, 15] demonstrated that putrescine can be formed from either arginine and ornithine in tobacco, and that tobacco roots fed with radiolabelled arginine readily incorporated it into nicotine [16]. However, from these experiments, the authors could not conclude which of the two pathways predominates. Our observations that: (i) specific inhibitors of ADC are more effective than an inhibitor of ODC in inhibiting alkaloid formation; and (ii) label from arginine is more efficiently incorporated into nicotine than label from ornithine suggest that ADC, not ODC, is the most important enzyme in the biosynthesis of putrescine-derived tobacco alkaloids.

EXPERIMENTAL

Plant material. Nicotiana tabacum L. cv. 'Wisconsin-38' and *Nicotiana tabacum* L. cv. 'Burley-21' plants were grown in plastic pots containing vermiculite, subirrigated twice daily with a 1.2 g/l soln of Hyponex [8] and maintained in a controlled growth room under a 16 hr light/8 hr dark photoperiod (9:1 energy mixture of fluorescent and incandescent light at 1.96 W.m^{-2}) at 24°.

Callus culture and addition of inhibitors. Explants were obtained from plants grown to the 17–20 leaf stage as previously described [8, 12]. Callus was grown on a modified Murashige–Skoog [17] medium containing 11.5 μM NAA, 1 μM kinetin and organic acids at 25° in the dark [12]. After 4 weeks, the calli were transferred to a medium like described above, but containing 1.5 μM NAA (optimal for alkaloid formation) [12] in the absence (control) or presence of different concn of inhibitors. Samples for growth measurements and alkaloid analysis were harvested at the sixth week of callus grown on the lower level of NAA.

Alkaloid analysis. Alkaloids were determined according to ref. [18]. Samples dried to constant weight at 60° for 24 hr were extracted with 20 vols of 25 mM K-Pi buffer (pH 7.8) at 30° for 24 hr with constant agitation. Alkaloids in the fresh tissue were extracted with 2 ml of 40% (v/v) MeOH containing 0.1% (v/v) HCl and then centrifuged at 500 g for 5 min. Both extracts were filtered through Whatman No. 2 filter paper and a 0.45 μm Millipore filter, diluted with H_2O, and injected into the HPLC system as a 20 μl aliquot. HPLC analysis was carried out with a programable Altex-Beckman model 322 liquid chromatograph, using a 4.6 × 250 mm 5 μm particle reverse-phase (C_{18}) column (Altex-octadecylsilane) and eluted with an isocratic mobile phase of 40% (v/v) MeOH containing 0.2% phosphoric acid buffered to pH 7.5 with triethylamine, at a flow rate of 1 ml/min at 254 nm.

Table 2. Incorporation of the radiolabelled precursors of putrescine into nicotine in the NTB-21 callus strain

Precursor	Incorporation ratio†	dpm/μmol nicotine	Nicotine (μmol/g fresh weight)
L-[U-^{14}C]Arginine	1.4	3900 ± 440*	7.1 ± 0.9
L-[U-^{14}C]Ornithine	0.5	1920 ± 140	4.6 ± 0.8

Numbers represent means ± s.e.m. of three replicates.

*Significantly different at the 5% level.

†Ratio in percentage of specific radioactivity of the nicotine to that of precursor fed.

Alkaloids were quantified with a 3370 A Hewlett–Packard integrator using nicotine (K & K Lab) and nornicotine (Roth) as standards.

Administration of the radiolabelled precursors of putrescine to the callus and isolation of nicotine. NTB-21 calli grown for 2 weeks on a low NAA level (1.5 μM) were aseptically transferred into Petri dishes (100 × 20 mm) with 6 ml of sterile H_2O containing 1 μCi of L-[U-^{14}C]Arg (288 mCi/mmol; ICN) or L-[U-^{14}C]Orn (266 mCi/mmol; NEN). After 6 hr of incubation in the dark at 30°, each callus was rinsed several times with H_2O in a shaker, dried on a filter paper and weighed. Alkaloids in the fresh tissue were extracted and analysed by HPLC as described above. From the chromatographic eluate aliquots corresponding to nicotine peaks were taken and dissolved in 4 ml Aquasol (NEN) for measuring radioactivity in a Beckman LS 7000 scintillation counter. Aliquots from the aq. medium were also measured for radioactivity at the start and end of the incubation period to evaluate the uptake by the callus of the two radiolabelled compounds.

Determination of arginase activity. Arginase activity was determined according to ref. [19] with few modifications. NTB-21 calli (3 g) were ground in 3 ml 0.1 M Tris-HCl buffer (pH 7.0) with 0.5% polyvinyl pyrrolidone, purified as described elsewhere [8], in chilled mortars. The extract was centrifuged at 15 000 *g* for 20 min and the supernatant fraction was used as the enzyme source. The reaction mixture contained 33 mM L-Arg (pH 9.6); 0.3 mol $MnCl_2$; glycine–NaOH buffer (pH 9.6); and enzyme preparation in a total volume of 1.5 ml. The mixture was incubated at 37° for 60 min. The reaction was stopped by adding 1 ml 10% (w/v) TCA and the protein was removed by centrifugation. A suitable aliquot of the supernatant fraction was used for urea estimation as described in ref. [19]. Urea standards were incubated with each set of assays. To evaluate possible interference, extracts of known amounts of bovine liver arginase (Sigma) were also added and the mixture analysed for enzyme activity.

Acknowledgements—We are grateful to R. Kaur-Sawhney and M. A. Masdéu for suggestions and help.

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