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CONJUGATION OF ASPARTIC ACID WITH 4-BROMOPHENYLACETIC ACID, AN AUXIN ANALOGUE OF ASPARTIC ACID

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Key Word Index—*Nicotiana tabacum*; Solanaceve; Tobacco; protoplasts; auxin; [^{14}C carboxyl]-4-bromophenylacetic acid; aspartic acid conjugate.

Abstract—Tobacco mesophyll protoplasts conjugate the auxins indoleacetic acid and naphthaleneacetic acid with aspartic acid very efficiently. This conjugation was found to be correlated with the toxicity of these molecules to protoplast-derived cells grown at low densities. Among a series of halogenated phenylacetic acids, 4-bromophenylacetic was toxic to cells grown at low densities although not able to stimulate proliferation at high cell densities, as opposed to indoleacetic acid and naphthaleneacetic acid. [^{14}C-carboxyl]-4-bromophenylacetic acid was conjugated with aspartic acid by tobacco protoplasts. Although 4-bromophenylacetic acid is not an auxin, this molecule shares with auxins some of their properties.

INTRODUCTION

Auxin conjugation processes are considered to play an important role in plant development by controlling the level of free growth regulators [1, 2]. Previous work has shown that structural analogues of indoleacetic acid such as naphthaleneacetic acid can be efficiently conjugated with aspartic acid, whereas other auxins such as picloram are not conjugated [3]. A correlation between auxin conjugation and auxin toxicity to cells plated at low densities was observed [3, 4]. The purpose of this work was to characterize the metabolism of a molecule structurally related to auxin, 4-bromophenylacetic acid.

RESULTS

A family of halogenated phenylacetic acids was investigated for its ability to promote growth and to induce toxicity at high concentration. Among these molecules, phenylacetic acids halogenated in the *meta* position, and to a lesser extent in the *ortho* position, were able to stimulate cell proliferation (Fig. 1). Phenylacetic acids halogenated in the meta position were toxic. When tested in the presence of growth stimulating concentrations of NAA, phenylacetic acids substituted in the para position with bromine or iodine, 4-bromophenylacetic acid and 4-iodophenylacetic acid, were found to be also toxic to cells grown at low densities (Fig. 1).

In a previous study on the metabolism of auxins, a correlation was observed between the toxicities of auxins to cells grown at low densities and the ability of the corresponding cells incubated at high cell densities to conjugate auxins with aspartic acid [3, 4]. It was therefore of interest to see whether this observation could be extended to 4-bromophenylacetic acid, a molecule structurally related to auxins which displays a toxic effect on cells grown at low densities. (^{14}C)-4-Bromophenylacetic acid was chemically synthesized from (^{14}C)-sodium cyanide as described in Experimental. Tobacco mesophyll protoplasts were incubated with [^{14}C]-4-bromophenylacetic acid or with [^{14}C]-NAA or [^{14}C]-picloram. As observed for [^{14}C]-NAA, [^{14}C]-4-bromophenylacetic acid was rapidly taken up by cells from the culture medium (Fig. 2). When extracted and analysed by thin layer chromatography, a major [^{14}C] metabolite derived from [^{14}C]-4-bromophenylacetic acid was detected, which displayed migratory properties similar to amino acid conjugates of auxin. A large amount (5 mg) of this metabolite was extracted from approximately 10^9 protoplasts incubated with 4-bromophenylacetic acid and further characterized by its mass spectrum. Main ions of *m/z* 357–359 were detected after methylation of the purified product and other peaks were also observed which correspond to the expected ionisation products of 4-bromophenylacetyl-L-aspartic acid. Although it was not growth stimulating, 4-bromophenylacetic acid was conjugated with aspartic acid by cells, as well as the toxic auxins NAA and IAA.

DISCUSSION

In previous studies, NAA was found to be required for low density growth of cells derived from protoplasts, this molecule being toxic at concentrations greater than 2 μM [6]. NAA was toxic for a variety of protoplast-derived cells grown at low densities, regardless of the tissue (mesophyll, epidermis, pith, callus) used as a source of protoplasts [7]. Among other auxins tested under similar growth conditions, IAA and, to a lesser extent, 2,4-D were found to be toxic, but picloram did not induce cell death, although it also promoted growth [7].

The heterogeneity of auxins as regards their toxicity, suggests that depending on their steric and electrostatic properties, they are more or less susceptible to induce this auxin-related cell killing. Since auxins like picloram are not cytotoxic it was expected that a structure–activity investigation could allow us to characterize molecules

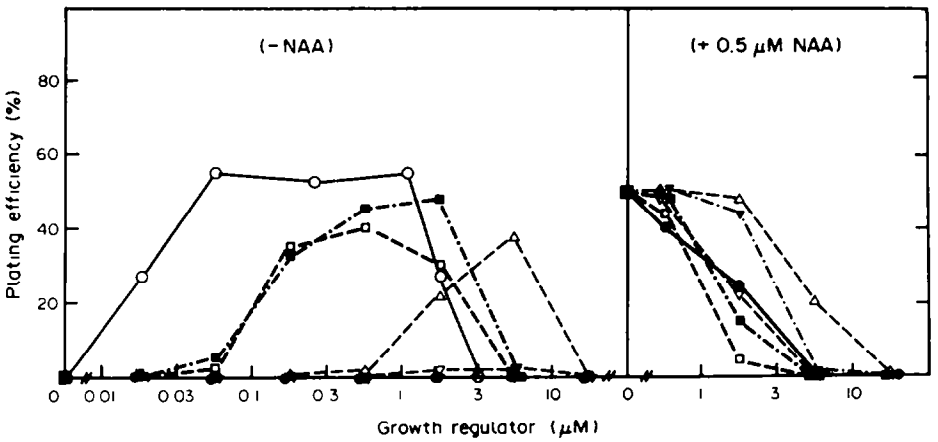

Fig. 1. Growth promoting activities and toxicities of halogenated phenylacetic acid. Protoplast-derived cells were incubated at low densities in the absence (– NAA) or presence of 0.5 μM NAA. Variable concentrations of 2-chloro (Δ), 3-chloro (□), 3-bromo (■), 4-chloro (▽), 4-bromo (▼) and 4-iodo (●) derivatives of phenylacetic acid were tested. A control with naphthaleneacetic acid alone (O) is also presented.

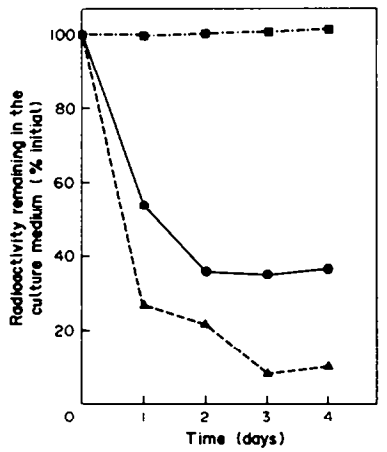

Fig. 2. Uptake of [^{14}C]-labelled auxins by tobacco mesophyll protoplasts. Protoplasts were plated at a density of 7×10^4 p-cells/ml in medium C cotaining (^{14}C)-NAA (15 μM, 0.5 μCi/ml), or (^{14}C)-picloram (15 μM, 0.5 μCi/ml) or a mixture of cold NAA (0.5 μM) and (^{14}C)-4-bromophenylacetic acid (15 μM, 0.5 μCi/ml). The radioactivity remaining in the culture medium was measured at various times after the beginning of incubation and expressed as a percentage of initial radioactivity in the culture medium. (O), (^{14}C)-NAA; (■), (^{14}C)-picloram; (▲), (^{14}C)-4-bromophenylacetic acid.

displaying only auxin-related toxicity. 4-Bromophenylacetic acid, a molecule which is not able to trigger cell proliferation, was toxic when tested in the presence of low, growth stimulating concentrations of NAA. Previous studies on auxin conjugation showed that this process was apparently correlated with auxin toxicity: the use of chemically synthesized [^{14}C]-4-bromophenylacetic acid allowed us to show that this molecule was conjugated with aspartic acid by protoplast-derived cells, as shown previously for NAA and IAA. We assume that this conjugation process is a detoxification reaction induced by cells incubated with toxic auxins. The observation that 4-bromophenylacetic acid can be recognized as an auxin, although it does not trigger cell proliferation, indicates that it shares certain characteristics with auxins.

EXPERIMENTAL

Protoplast isolation and culture. Nicotiana tabacum (c.v. *Xanthi*) plants used as the source of protoplasts were grown in the greenhouse as previously described [5]. The procedures for mesophyll protoplasts isolation, culture of protoplasts at high density in medium To, and culture at low density in medium C of protoplast-derived dividing cells (p-cells) obtained after 4 days of incubation of protoplasts at high density are described in ref. [6].

Test for auxin stimulation. The cells derived from protoplasts were washed twice in medium C depleted of NAA and diluted in the same liquid medium to a final density of 100 p-cells/ml. Variable concs of auxin were added to the culture medium, and triplicate samples of 5 ml were incubated for one month at 26° under 16 hr light per day (10 W/m^2). Plating efficiencies (PE), i.e. the ratio of visible colonies to initially plated p-cells were calculated. Standard deviations of PE measurements did not exceed 10% of the calculated plating efficiencies.

Incubation with labelled NAA and extraction of amino acid-auxin conjugates. [1-^{14}C]-NAA (62 Ci/mol) was obtained from Amersham, England. [1-^{14}C]-picloram (40 Ci/mol) was a gift of Claude Martin, I.N.R.A.-Dijon. The procedure for incubation of p-cells in the presence of [^{14}C]-auxin, EtOH extraction of metabolites from pelleted cells, TLC of concd extracts on silica gel plates with organic solvents ($CHCl_3$–MeOH: HOAc, 15:4:1), and autoradiography have been described in ref. [3].

Chemical synthesis of [^{14}C-carboxyl]-*4-bromophenylacetic acid.* Sodium [^{14}C]-cyanide (sp. act., 43–44 mCi/mmol) was purchased from C.E.N (Saclay, France). Radiochemical purity was checked on a Berthold apparatus and by autoradiography. The classical scheme of the synthesis is indicated below [7]:

$$1\ p\mathrm{Br\,C_6H_4CH_2Br} \xrightarrow{Na^{*}CN} p\mathrm{Br\,C_6H_4CH_2{}^{*}CN}\ 2$$

$$\xrightarrow{NaOH} p\mathrm{Br\,C_6H_4CH_2{}^{*}COOH}\ 3$$

The condensation between 4-bromobenzylbromide 1 and labelled NaCN (sp. act. 43–44 mCi/mmol) was achieved by boiling in aq. EtOH for 6 hr. The nitrile 2 was hydrolysed *in situ* by boiling in ethanolic NaOH. After acidification, the 4-bromophenylacetic acid 3 crystallizes from the soln. The yield was 90%. TLC on silica gel HF 254 utilizing Et_2O as the eluent showed a single fluorescent spot. The identity of the final product with [^{14}C-carboxyl]-*p*-bromophenylacetic acid was confirmed by mass spectrometry (sp. act., 7.17 mCi/mmol; radioactive yield: 95.6%).

Analytical procedure and spectroscopy. Proton NMR spectra were recorded on a Varian T.60 spectrometer. The [^{13}C] NMR spectra were recorded on a Varian CFT.20 spectrometer operating at 20 MHz in a Fourier transform mode. Samples were dissolved in $CDCl_3$ containing 1% TMS as an int. standard. IR spectra were recorded on a Perkin-Elmer 257 spectrometer. Samples were dissolved in CCl_4 or $CHCl_3$. Microanalyses were performed at the Central Microanalysis Laboratory, C.N.R.S., Gif-sur-Yvette, France. Mass spectra were recorded on an ATLAS CH_7 spectrometer.

4-bromophenylacetonitrile 2. Mp 45–47°. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 3250, 2980, 1600, 1495, 1415, 1075 and 1015. ^{1}H NMR; δ7.30 (4H, C_6H_4), 3.66 (2H AB system, J_{AB} = 5 Hz, CH_2) ^{13}C NMR: 132.7 and 129.3 (C_6H_4), 122.2 (C≡N), 23.2 (CH_2).

4-bromophenylacetic acid 3. Mp 115–117°. IR ν^{CHCl_3} 3600–2500, 1715, 1480, 1410 and 1070. ^{1}H NMR: 7.22 (4H C_6H_4) 3.50 (2H AB system, J_{AB} = 5 Hz, CH_2) ^{13}C NMR: 177.6 (COOH), 132.2–121.6 (C_6H_4), 40.4 (CH_2).

Mass spectrometry of [^{14}C-carboxyl]-4-bromophenylacetic acid and its conjugate with aspartic acid. These spectra were obtained on a Atlas CH_7 spectrometer. The labelled 4-bromophenylacetic acid is characterized by ions at *m/z* 214 (43.1), 216 (48.3) and 218 (6.3), other brominated fragments $[BrC_7H_6]^+$ 169 (100), 171 (97.6), and $[C_7H_6]^+$ 90 (55.4), 91 (37.3) and 89 (45.6).

The conjugate with aspartic acid after isolation and reaction with CH_2N_2 is characterized by ions at *m/z* 357 (9.7), 359 (10.8), other brominated fragments $[BrC_6H_4CH_2CONHCHCH_2COOCH_3]^+$ 298 (7.1), 300 (6.1), $[BrC_6H_4CH{=}C{=}O]^+$ 196 (7.7), 198 (8.0), $[BrC_7H_6]^+$ 169 (43), 171 (42.9) and $[C_{10}H_{10}NO]^+$ 160 (89.5). The peak (100) corresponds to 102, $[C_4H_8NO_2]^+$.

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