BIOTRANSFORMATION OF 2-(p-METHOXYPHENYL) ETHYLAMINE BY *CATHARANTHUS ROSEUS* AND *STROBILANTHES DYERIANUS* CELL CULTURES

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ABSTRACT.—The metabolism of several 2-phenylethylamines was examined in five plant cell cultures. Metabolites were produced only in *Catharanthus roseus* and *Strobilanthes dyerianus* cultures with 2-(*p*-methoxyphenyl) ethylamine (**1**) as the substrate. The former culture demethylated the substrate to form 2-(*p*-hydroxyphenyl) ethylamine (**2**), and the latter culture metabolized it to 2-(*p*-methoxyphenyl) ethyl-*β*-D-glucopyranoside (**3**), a new derivative of 2-(*p*-methoxyphenyl) ethyl alcohol (**4**).

Recent investigations have shown that plant cell suspension cultures are useful systems for studying the metabolism of various compounds and that they have the capability to catalyze many types of reactions (1). However, little is known about the metabolism of aromatic amines in plant cell cultures (2, 3, 4). The objective of the present research was to study the metabolism of selected phenylethylamines and related compounds by plant cell suspension cultures.

EXPERIMENTAL[1]

TISSUES AND THEIR SOURCES.—Five tissue cultures were employed in this study. These cultures included *Apocynum cannabinum* L., *Catharanthus roseus* (L.) G. Don, *Conium maculatum* L., *Strobilanthes dyerianus* Hort. and *Ruta graveolens* L. The *A. cannabinum*, *C. roseus* and *C. maculatum* static cultures were maintained as previously reported (5, 6). *R. graveolens* cultures were maintained on PRL agar medium (7) under continuous fluorescent light; these cultures were white to green in color and partially differentiated. The *S. dyerianus* tissue was initiated in 1976 by Dr. R. J. Krueger and maintained on PRL agar media. All of the static cultures (except *Ruta*) were grown in the dark at 25° and subcultured to fresh agar media every four to five weeks. Suspension cultures were initiated from the above static cultures by transferring them to the corresponding liquid media. These cultures were grown on rotary shakers (90–125 rpm) in the dark at 25° in cotton-plugged 250 ml Erlenmeyer flasks containing 50 ml of medium. All (except *S. dyerianus*) were transferred to fresh media every three weeks. The *S. dyerianus* tissue was transferred every two weeks.

SUBSTRATES AND CONDITIONS USED.—Substrates used were 2-(*p*-hydroxyphenyl) ethylamine hydrobromide, 2-(*p*-methoxyphenyl) ethylamine hydrochloride, amphetamine sulfate, *p*-hydroxyamphetamine hydrobromide, *p*-methoxyamphetamine hydrochloride, 1-(*p*-hydroxyphenyl)-2-aminobutane hydrochloride and 1-(*p*-methoxyphenyl)-2-aminobutane hydrochloride. The first three substrates were dissolved in 70% ethanol; the last four were dissolved in dimethylformamide (DMF) as vehicles for addition to cell incubation. No obvious harmful effects on cell growth were observed with these vehicles. Solutions of 15 mg substrate/0.2 ml were added to each 250 ml flask containing 14-day old cell suspension tissue. Substrate, cells and medium were then incubated for periods of 1, 3, 7, and 14 days. At the end of those periods, the contents of two flasks for each substrate and plant cell culture were harvested and processed

[1]Melting points were determined in a Thomas Hoover capillary melting point apparatus and are uncorrected. Molecular weights were determined by the Rast method (8), using camphor as a solid solvent. Nuclear magnetic resonance (NMR) spectra were obtained with a Varian T-60 spectrometer, using TMS as an internal standard and chemical shifts reported in δ (ppm) units. Elementary analyses obtained from Galbraith Infrared spectra (KBr) were recorded with a Beckman IR 4240 spectrophotometer. Mass spectra were run on a Finnigan model 3200 mass spectrometer. Ultraviolet spectra were obtained with a Phillips Pye-Unicam SP 1800 ultraviolet spectrophotometer. Optical rotations were measured on a Perkin-Elmer 141 polarimeter.

as described below. Controls consisted of cultures grown without substrate or substrate suspended in culture media without cells.

TLC ANALYSIS. –Extracts were subjected to tlc analysis on 0.25 mm layers of silica gel GF_{254} (Merck) on glass plates. Prior to use, tlc plates were activated at 110° for 1 hr. Solvent systems used in developing the plates were: A, chloroform-methanol-90% formic acid (10:2:1); B, chloroform-acetone-methanol-28% ammonia (70:25:4:1); C, *n*-butanol, acetic acid, water (4:1:1); D, chloroform-methanol (95:5). After development, the plates were examined under 254 nm and 365 nm ultraviolet light. They were then sprayed separately with ninhydrin reagent (9) and *p*-anisaldehyde reagent (9) and heated. Thin layer chromatographic analysis of extracts revealed that only 2-(*p*-methoxyphenyl) ethylamine (**1**) was appreciably metabolized and by only two tissues, *C. roseus* and *S. dyerianus*. Therefore, experiments were conducted with these tissues and 2-(*p*-methoxyphenyl) ethylamine in order to isolate and identify the metabolite(s) formed.

BIOTRANSFORMATION OF 2-(*p*-METHOXYPHENYL) ETHYLAMINE BY *C. roseus* SUSPENSION CULTURES.—Preliminary experiments showed that a 1–2 week incubation period of substrates and tissues gave optimum yields of metabolites. Fifty mg of 2-(*p*-methoxyphenyl) ethylamine hydrochloride, dissolved in 0.5 ml of 70% ethanol, was added to each of forty 500 ml flasks containing 100 ml of medium and 3.4 g (wet weight) of 10-day old *C. roseus* suspension tissues. The cultures with substrates were incubated on a shaker for 11 days. At the end of that time, the cells were separated from the media by filtration. The cells (689 g fresh weight) were homogenized in a Waring Blendor with 800 ml of ethyl acetate. A second homogenation was carried out with 400 ml of ethyl acetate. The homogenate was stirred for 2 hours and filtered. After the ethyl acetate layer was removed, the aqueous layer was adjusted to pH 11 and extracted twice with 200 ml volumes of ethyl acetate. The ethyl acetate layer was removed and dried over anhydrous sodium sulfate. The solvent was evaporated to produce 0.17 g of extract which was further purified by preparative tlc in solvent system A. The medium from the 11 day incubation of substrate and *C. roseus* suspension tissue was adjusted to pH 11 and extracted with ethyl acetate; this extract was concentrated to dryness (.14 g). No metabolites were found in this medium extract.

BIOTRANSFORMATION OF 2-(*p*-METHOXYPHENYL) ETHYLAMINE BY *S. dyerianus* SUSPENSION CULTURES.—Preliminary experiments here showed that a 7-day incubation period gave optimum yields of metabolites. In an effort to further study the metabolism of **1**, a total of 1.9 g of 2-(*p*-methoxyphenyl) ethylamine hydrochloride was added to 10-day-old *S. dyerianus* cell suspension cultures contained in thirty-eight 500 ml Erlenmeyer flasks. Cells and medium were harvested after 7 days and separated by filtration. No metabolites were found in the filtrate. The cells (687 g fresh weight) were homogenized twice with 1.4 liter and 0.7 liter of ethyl acetate and stirred for 2 hours each time. The homogenate was filtered and the cell residue was reextracted with 400 ml of methanol to yield a methanol extract weighing 2.02 g (Fraction A). The ethyl acetate layer, after drying over anhydrous sodium sulfate, was concentrated to dryness and had a weight of 1.49 g (Fraction B). Fractions A and B were separately subjected to preparative tlc (0.75 mm thick silica gel GF_{254}) in solvent system A. One major spot at Rf 0.4 was scraped off and eluted with chloroform-methanol (1:1), and the extract was concentrated to dryness. The crude metabolites (0.4 g from fraction A and 0.2 g from fraction B) were crystallized as white granules from ethyl acetate-hexane and recrystallized from ethyl acetate. Physical properties of the metabolites isolated from both fractions A and B showed them to be identical. They had the following properties: mp 115–6°; molecular weight (Rast method) 316±13; $[\alpha]^{25}D$ −28° (c 2.0, MeOH); uv, λ max (MeOH), 278 (log ε 3.23), 284 (3.17), no shift with KOH; ir (KBr), 3530, 3430, 3230, 1610, 1510, 1440, 1370, 1345, 1320, 1300, 1240, 1160, 1125, 1110, 1090, 1070, 1025, 890, 815 cm^{-1}; nmr (MeOH-d_4) δ 2.83 (t, 2H, Ar-CH_2), 3.73 (s, 3H, OCH_3), 3.2–4.4 (*m*, 13H, Ar-CH_2-CH_2 and sugar protons), 6.73 (d, 2H, J=8 Hz, Ar*H*), 7.10 (d, 2H, J=8 Hz, Ar*H*); ms, *m/e* (%): 314 M^+ (1), 180 (1), 152 (4), 135 (92), 134 (100), 122 (6), 121 (40), 120 (6), 119 (6), 103 (6), 91 (11), 77 (6); Anal. calcd. for $C_{15}H_{22}O_7$: C, 57.32; H, 7.01. Found: C, 57.55; H, 7.25. Nmr (pyridine-d_5) of the compound gave signals based on the glucosyl proton at 4.80 (J=7 Hz). The metabolite was hydrolyzed with 1% β-glucosidase in 0.1 M acetate buffer (pH 5.1) for 2 hours at 37° or by refluxing with 1 N HCl for 30 min. A glucose enzymatic test strip (U.S.P.) was used for the detection and estimation of approximate concentration of glucose in the hydrolysate. Identification of glucose was confirmed by tlc on silica gel GF_{254} in a solvent system of *n*-butanol-acetic acid-ether-water (9:6:3:1). Paper chromatography was also employed with three solvent systems which were *n*-butanol-benzene-pyridine-water (5:1:3:3), *n*-butanol-ethanol-water (4:1:2.2) and *n*-butanol-acetic acid-water (4:1:5, top layer). The aglycon was isolated from the hydrolysate and exhibited physical properties (tlc, ir and ms) identical to those of an authentic sample of 2-(*p*-methoxyphenyl) ethyl alcohol (**4**).

BIOTRANSFORMATION OF 2-(*p*-METHOXYPHENYL) ETHYL ALCOHOL TO ITS GLUCOSIDE BY *S. dyerianus* CELLS.—A total of 150 mg of **4** was incubated with 15-day-old *S. dyerianus* cell suspension cultures contained in ten 250-ml Erlenmeyer flasks. The cultures were harvested after 6

days and analyzed by a method similar to that described for the isolation of the biotransformation product of **1** by *S. dyerianus* cells. A metabolite was isolated from ethyl acetate and methanol extracts and was identified (tlc, mmp, ir, ms) as 2-(*p*-methoxyphenyl) ethyl β-D-glucopyranoside (**3**) by comparison with a sample isolated in the previous experiment.

RESULTS AND DISCUSSION

SCREENING.—When cell suspension cultures of five different plant species were incubated with seven substrates consisting of 2-phenylethylamines and related 2-phenylamines, extraction and chromatographic analysis showed that some substrates remained in the media while others were taken up by the cells. Suspension cultures of *A. cannabinum*, *C. roseus* and *R. graveolens* took up more of the various substrates than cells of *C. maculatum* and *S. dyerianus*. Para-hydroxyamphetamine was almost completely absorbed by the cultured cells of *C. roseus* and *R. graveolens*. Cultures used as controls, as previously described, did not exhibit the ability to form any metabolites.

Of the several substrates fed to cultures, only **1** was metabolized; this was accomplished only by *C. roseus* and *S. dyerianus* cells. The metabolite, which was extracted from *C. roseus* cells and detected under uv light, produced a purple color with ninhydrin reagent. It was suspected that the metabolite was **2**, and this proved to be true when the metabolite was co-chromatographed with a pure sample of **2**. Demethylation of codeinone by *Papaver somniferum* cells has been previously demonstrated (1).

ISOLATION AND IDENTIFICATION OF 2-(*p*-METHOXYPHENYL) ETHYL β-D-GLUCOSIDE.—When ten-day-old *S. dyerianus* cell suspension cultures were incubated with **1** and later extracted, both the ethyl acetate and methanol extracts contained a metabolite. Separate physical and chemical analyses showed them to be identical. The metabolite exhibited negative reactions with ninhydrin and Dragendorff reagents. Elemental analysis showed the absence of nitrogen. While the substrate was optically inactive, the metabolite was optically active. The uv spectrum showed λ max (MeOH) 278 nm (log ε 3.23) and 284 (3.17) and was similar to that of the substrate. Nmr spectral data indicated the presence of a methoxy group at δ 3.73 and four aromatic protons at 6.73 and 7.10. A triplet at 2.83 (2H) indicated the presence of an $-CH_2CH_2-$ group. Those data demonstrated that the *p*-methoxyphenylethyl moiety was still intact. The ir spectrum (KBr) showed strong broad bands at 3530–3230 (OH) and 1125–1025 (>CH–O, $-CH_2O$), which are typical of sugars. The estimation of molecular weight by the melting point depression method was carried out to confirm the molecular ion in the mass spectrum which showed several small peaks over *m/e* 135. The estimated molecular weight of 316±13 revealed *m/e* 314 to be the molecular ion. The above data suggested that the metabolite might be a glycoside of deaminated substrate. The metabolite was hydrolyzed by acid and by β-glucosidase. Glucose was identified in the hydrolysate by paper and thin layer chromatography. The aglycone was identified as **4** by co-chromatography and the comparison of ir and mass spectra with those of an authentic sample. The nmr spectrum of the metabolite in pyridine-d_6 gave peaks of glucosyl H–1 at 4.80, $J_{1,2}=7.0$ Hz, indicating it was β-D-glucopyranoside (10). Thus, the novel metabolite was confirmed as 2-(*p*-methoxyphenyl) ethyl β-D-glucopyranoside. The yield of the metabolite was 20%, based on isolated crude crystalline material. As expected, the same metabolite, 2-(*p*-methoxyphenyl) ethyl glucoside, was isolated from *S. dyerianus* cell suspension cultures which were fed **4**, the presumed intermediate of 2-(*p*-methoxyphenyl) ethyl glucoside.

The present findings suggest that conversion of an amine to alcohol and glucosylation took place consecutively during the incubation of **1** with *S. dyerianus* cell suspension cultures. Each of those reactions is common to the metabolism of foreign compounds administered to plant cell cultures. The conversion of aromatic amines to carboxylic acids or alcohols is known from animals (11) and microorganisms (12, 13, 14) as well as plant cells (1). Conjugation of exogenous compounds with carbohydrates, amino acids, fatty acids, or other endogenous metabolites proceeds quite often in plant cells (1,3). Conjugation with glucose appears to occur most frequently (3). Meyer and Barz (4) studied the degradation of ^{14}C-labelled hordenine, tyramine and dopamine in barley and several other plant cell suspension cultures and found that intermediates of catabolic pathways seem to be withdrawn from metabolism by conjugation reactions. The present results suggest that some of those conjugates might be glycosides of catabolic intermediates.

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