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Plant Metabolic Studies of the Growth Regulator Maleic Hydrazide

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The metabolism of maleic hydrazide has been studied in cell suspension cultures of soybean, wheat, and maize under standardized conditions (40 mL flasks, 1 ppm, 48 h). Maleic hydrazide was converted to its β-D-glucoside as the predominant soluble metabolite in yields of between 2 and 15%. The latter was completely cleaved under simulated stomach conditions (pH 1, 37 °C, 24 h). In addition, up to 18% of the applied maleic hydrazide became associated with the nonextractable residue. The residue from soybean cells was solubilized only to a low degree (~3%) under simulated stomach conditions. The lignin and hemicellulose components appeared to contain most of the radioactivity in the nonextractable residue from soybean cells. It is concluded that metabolism in cultured plant cells resembled that in whole plants and that the β-D-glucoside of maleic hydrazide belongs to the small group of acid-labile pesticidal conjugates.

Keywords: *Maleic hydrazide; cell suspension cultures; soybean; wheat; maize; bound residues; β-D-glucoside; acid-labile*

INTRODUCTION

Maleic hydrazide (MH) is a plant growth regulator and herbicide applied mainly to tobacco, potatoes, and onions. Besides its many physiological side activities, it acts as an antagonist of pyrimidine bases (Appleton et al., 1981; Weed Science Society of America, 1989). MH is freely translocated in plants, with mobility in both phloem and xylem (Meyer et al., 1987). Mutagenic properties of MH were documented in plants (Plewa and Wagner, 1981, 1993; Gichner et al., 1992) but not in animals (Meyer et al., 1987). With regard to plant metabolism, it has been stated that MH becomes fixed within the plant and is not metabolized (Weed Science Society of America, 1989). However, MH β-D-glucoside (MHG) had previously been found to be formed in 15% yield in wheat leaf segments (Towers et al., 1958). The conversion of MH to its β-D-glucoside is depicted in Figure 1.

A thorough study of MH metabolism in tobacco plants has shown that the parent compound declined by 85% over 3 weeks, with conversion to nonextractable and extractable conjugates (Frear and Swanson, 1978). A similar metabolite pattern was obtained with American elm seedlings (Domir, 1980). The main component in the soluble conjugate fraction of tobacco was identified as the β-D-glucoside (Frear and Swanson, 1978). A significant incorporation of MH into the nonextractable residue of tobacco also occurred, apparently mainly into the lignin fraction (Frear and Swanson, 1978; Meyer et al., 1987). Formation of bound MH residues was confirmed for other plant species, such as American elm (Domir, 1980) as well as corn and pea seedlings (Nooden, 1970). Upon acid or base hydrolysis, or heating with 2-aminoethanol, MH was released as a main (20–60%) product from the nonextractable residues (Frear and Swanson, 1978; Nooden, 1970). It was proposed that free and bound MH may represent a serious human hazard in plant food materials (Nooden, 1970).

In view of the unusually simple reported whole-plant MH metabolism, it has now been examined whether the MH metabolite pattern can be reproduced in a standardized plant cell culture test previously described (Sandermann et al., 1984; Komoβa et al., 1992). In addition, the β-D-glucoside and the nonextractable residue formed from MH were exposed to simulated stomach pH conditions as a simple first test of potential animal bioavailability.

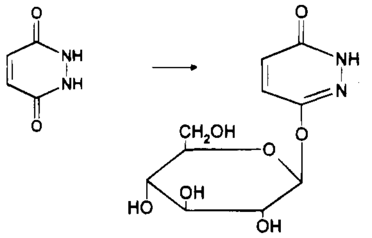

Figure 1. Conversion of maleic hydrazide to its *O*-β-D-glucoside.

MATERIALS AND METHODS

Chemicals. [2,3-^{14}C]Maleic hydrazide (specific activity 9.3 MBq/mg; radiochemical purity >99% as determined by HPLC, see below) was obtained from Pathfinder (St. Louis, MO), whereas nonlabeled MH was from Riedel-de Haën (Seelze, Germany). MH β-D-glucoside (MHG) was synthesized according to the procedure of Newsome (1980). All other chemicals were of analytical grade.

Plant Cell Cultures. Cell suspension cultures of soybean (*Glycine max* L. Merr. cv. Mandarin) and wheat (*Triticum aestivum* L. cv. Heines Koga II) were grown in B5 medium (designated B5), and maize (*Zea mays* L. cv. Black Mexican Sweet) was grown in modified MS medium (designated mMS). The growth media and techniques used have been previously described (Komossa et al., 1992). Different cell lines of soybean and maize designated B5M and mMSM, respectively, were cultivated in slightly modified media with 0.25 mg/L (rather than 2.5 mg/L) of $CuSO_4$ and $CoCl_2$. Sterility controls were performed as earlier described (Komossa et al., 1992).

Determination of Radioactivity. The determination of radioactivity in solution, in HPLC eluates, in $^{14}CO_2$, and in bound residues was carried out as described (Komossa et al., 1992).

HPLC Analysis. A Beckman chromatograph (Munich, Germany) was used as described (Komossa et al., 1992). Separation of MH and MHG was performed by modification of a published method (Newsome, 1980), using a Spherisorb

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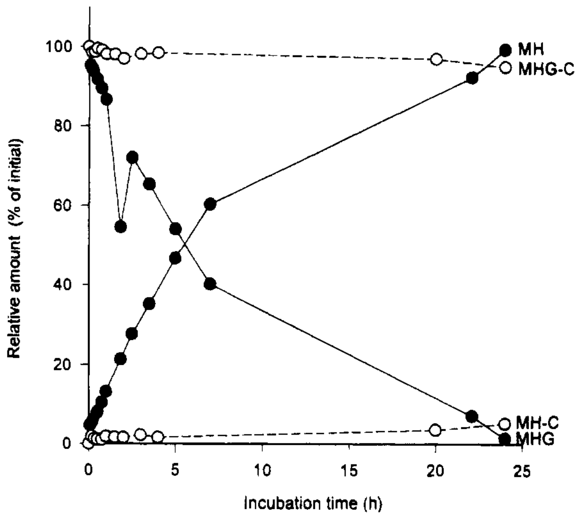

Figure 2. Hydrolysis of the β-D-glucosyl conjugate of maleic hydrazide in distilled water (○; control) and in 0.1 M HCl, 37 °C (●). C indicates control.

SAX column (250 × 4.6 mm, 5 μm) (Bischoff, Leonberg) with 9 parts of 10 mM sodium formate (adjusted to pH 4.5 with formic acid) and 1 part of acetonitrile as eluent. The flow rate was 1 mL/min, and UV detection was at 313 nm. The retention times of the reference standard compounds were 5.6 min for MH and 2.8 min for MHG.

Metabolism in Cell Cultures. Incubation and extraction of the plant cell cultures were performed according to procedures slightly modified from those in the literature (Sandermann et al., 1984; Komossa et al., 1992). Filter-sterilized [2,3-^{14}C] MH (37 kBq; 1 ppm, corresponding to 8.9 μM) was added in 40 μL of methanol to 40 mL of culture medium on the 5th day of the growth period for soybean and on the 12th day for wheat and maize cultures. After an incubation period of generally 48 h, the growth medium was separated from the cells by vacuum filtration. The cells were suspended in 80% aqueous methanol at 4 °C and ground in a mortar for 10 min. After filtration, the cell material was again ground in 80% methanol for 10 min. The combined homogenates were centrifuged (40000*g*, 30 min, 4 °C), and the supernatant was concentrated at reduced pressure for HPLC analysis. The pellet was extracted sequentially with methanol, water, methanol, and water. The material was then lyophilized and ground to a powder by means of a Dismembrator instrument (Braun, Melsungen, Germany). The powdered material was sequentially extracted with dichloromethane/methanol/water (1:2:0.8) and 1% (w/v) aqueous sodium dodecyl sulfate, washed with water, and finally freeze-dried and again ground to a powder using the Dismembrator instrument. The final material represented the nonextractable residue fraction.

Fractionation of Soybean Bound Residue. A portion (1 g, 15.4 kBq) of the nonextractable residue from soybean B5 cultures was fractionated according to the procedure of Langebartels and Harms (1985), as modified by Komossa et al. (1992). The residue was sequentially treated with α-amylase (24 h), Pronase E (24 h), pectinase (24 h), dioxane/water 9:1 (v/v; 24 h), dioxane/2 N HCl 9:1 (v/v; 27 h), 24% (w/v) KOH (26 h), and 72% (v/v) H_2SO_4 (6 h). In independent experiments, 98 mg (1.5 kBq) of the soybean B5 nonextractable residue was incubated in 10 mL of 2-aminoethanol at 175 °C for 2 h [according to the procedure of Nooden (1970)]. After cooling, the solution was adjusted to pH 1 with about 15 mL of 5 M HCl, and the amount of solubilized radioactivity was determined.

Mild Acid Hydrolysis. An aliquot (1 g, 15.4 kBq) of the nonextractable soybean B5 residue was incubated in 30 mL of 0.1 N HCl at 37 °C for 24 h (Sandermann et al., 1992). A 0.5 mL sample was withdrawn, centrifuged, and analyzed for released radioactivity. The final supernatant was analyzed by HPLC. In addition, 0.5 g (7.8 kBq) of the bound soybean B5 residue was incubated in 30 mL of dioxane/0.1 N HCl (4:1 v/v) at 37 °C for 24 h and analyzed by using the same procedure. For examination of the soluble MHG, 1 mg of the synthetic reference compound was dissolved in 1 mL of either 0.1 N HCl or water and incubated for 24 h at 37 °C. At the time intervals plotted in Figure 2, 20 μL samples were withdrawn, diluted 1:50 (v/v) with water, and immediately analyzed by HPLC.

RESULTS

Metabolism in Cell Cultures. A standardized test procedure for plant cell suspension cultures (Sandermann et al., 1984; Komossa et al., 1992) was used with three plant species. The distributions of radioactivity among the different fractions of the workup procedure are summarized in Table 1. HPLC of the growth medium showed the presence of only MH. In the cell extracts, MH predominated, but, in addition, significant amounts (2–7% in wheat and maize and up to 15% in soybean) of MH β-D-glucoside were present. The latter was identified by its exact coelution with synthetic reference compound upon HPLC.

The soybean B5 residues from Table 1 were subjected to a published cell wall fractionation procedure (Langebartels and Harms, 1985; Komossa et al., 1992), as summarized in Table 2. Most of the nonextractable radioactivity was solubilized upon treatment with dioxane/HCl (operationally defined as lignin fraction; 30% of initial ^{14}C) and KOH (operationally defined as hemicellulose fraction; 13% of initial ^{14}C). A high amount (22% of initial ^{14}C) remained undigested even after the treatment steps employed. The harsh solubilization procedure of Nooden (1970) (2-aminoethanol, 175 °C, 2 h) released 28% of the bound radioactivity.

Test of Acid Stability. Treatment of the soybean B5 residue under simulated stomach conditions [0.1 M HCl, 37 °C, 24 h; cf. Sandermann et al. (1992)] released only 3% of the bound radioactivity. When dioxane/0.1 M HCl (4:1 v/v) was employed, the release rate was 4.5%. In both cases, 80–90% of the released ^{14}C cochromatographed with the MH standard.

MH β-D-glucoside is known to be cleaved by strong acid (1 or 2 N HCl, 100 °C; Towers et al., 1958; Frear and Swanson, 1978; Domir, 1980). Simulated stomach

Table 1. Maleic Hydrazide Metabolism in Plant Cell Suspension Cultures

	radioactivity (%)				
cell culture	growth medium	cell extract (MHG)	$^{14}CO_2$	nonextractable residue	recovery
soybean B5M	42.1 ± 1.8	29.5. ± 2.3 (14.0)	nd	18.3	89.9 ± 1.7
soybean B5	72.8 ± 5.2	9.7 ± 0.5 (15.0)	0.5 ± 0.1	9.7	92.8 ± 4.9
wheat B5M	61.1 ± 1.5	39.1 ± 4.3 (2.2)	nd	2.6	102.8 ± 5.4
maize mMSM	44.1 ± 3.2	60.9 ± 3.0 (5.1)	nd	0.2	105.2 ± 5.2
maize mMS	41.9 ± 0.4	61.1 ± 1.5 (6.8)	nd	0.1	103.1 ± 1.7

[a] The cell cultures and culture conditions used are indicated. The percent distribution of radioactivity after an incubation period of 48 h with 1 ppm [2,3-^{14}C]MH is shown for culture medium, cell extract, $^{14}CO_2$, and nonextractable (bound) residue. Each incubation was performed in four replicates, except for soybean B5 with 12 replicates. The standard deviations are indicated except for the nonextractable residues, for which standard deviations could not be calculated because the residues of each series were combined. The percentage of MHG in the cell extract is given in parentheses. It was estimated by HPLC. [b] nd, not determined.

Table 2. Cell Wall Fractionation of the Soybean B5 Nonextractable Residue (See Table 1) According to the Procedure of Langebartels and Harms (1985)[a]

treatment	radioactivity (%)	treatment	radioactivity (%)
α-amylase	7.2	KOH	13.8
Pronase E	8.9	H_2SO_4	4.4
pectinase	4.2	final residue	22.1
dioxane/H_2O	1.0		
dioxane/HCl	30.8	total	92.4

[a] The treatment applied and the percentage of initial radioactivity solubilized in each step are shown. The experiments were performed exactly as described (Komossa et al., 1992).

conditions (0.1 M HCl, 37 °C) sufficed to cleave MH β-D-glucoside completely within 24 h, as shown in Figure 2. A control incubation in distilled water gave only 1–2% cleavage after 24 h.

DISCUSSION

The present cell culture results confirm previous metabolic studies with wheat leaf segments (Towers et al., 1958), intact tobacco plants (Frear and Swanson, 1978), and American elm (Domir 1980) as well as corn and pea seedlings (Nooden, 1970). MH again had a relatively simple metabolic pattern giving rise mainly to the β-D-glucoside shown in Figure 1 and to nonextractable residues. The present results again demonstrate that metabolism in intact plants can be reproduced with standardized cell suspension culture systems. In further agreement with previous results (Frear and Swanson, 1978; Meyer, 1987) the cell wall lignin component appeared to be one of the binding sites in the nonextractable residue.

It was proposed earlier that MH bound in plant food materials could be a serious human hazard (Nooden, 1970). However, under simulated stomach conditions (0.1 N HCl, 37 °C, 24 h) the nonextractable soybean residue had only a low release rate (3–4%). The β-D-glucoside was found to be completely split under such mild conditions. Most *O*-glucopyranosides possess relatively high acid stability (Overend, 1972), although the bound aglucones usually become bioavailable through the action of intestinal β-glucosidases (Edwards and Hutson, 1986). Certain *N*-glucosides have previously been found to be completely cleaved under simulated stomach conditions (Sandermann, 1987; Winkler and Sandermann, 1989). The acid sensitivity of these *N*-glucosides has been thoroughly characterized with regard to rate and equilibrium constants (Winkler and Sandermann, 1992). Such studies have not yet been performed for maleic hydrazide. MH β-D-glucoside can isomerize back to the hydrazide tautomer (see Figure 1). This structural feature is proposed here as a partial explanation of acid sensitivity, in analogy to the acid sensitivity of phosphoenolpyruvate. Together with acid-labile linkages in enzymatic chloroaniline/lignin conjugates (Sandermann et al., 1992), there are now several examples for acid-labile pesticidal plant conjugates. Animal bioavailability of pesticidal conjugate metabolites generally is a prerequisite for toxicological effects (Edwards and Hutson, 1986; Sandermann, 1987).

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