Biotransformation of Pyrene by Cell Cultures of Soybean (*Glycine max* L.), Wheat (*Triticum aestivum* L.), Jimsonweed (*Datura stramonium* L.), and Purple Foxglove (*Digitalis purpurea* L.)

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The metabolism of the four-ringed polycyclic aromatic hydrocarbon (PAH) pyrene was investigated using cell suspension cultures of soybean, wheat, purple foxglove, and jimsonweed and callus cultures of soybean and foxglove. In all species, nonextractable residues were found (soybean, jimsonweed, and foxglove suspensions, <10% of applied ^{14}C; soybean and foxglove callus cultures, 20−25%; wheat, 30−40%); soluble metabolites were detected in only foxglove and wheat. About 90% of applied pyrene was transformed in wheat. Corresponding data from soybean and foxglove callus cultures were about 30% and those from soybean, jimsonweed, and foxglove suspensions about 7%. In foxglove, 1-hydroxypyrene methyl ether was identified as the main metabolite, whereas a complex mixture of carbohydrate conjugates of 1-hydroxypyrene was found in wheat. Due to the present results, crop and wild plants may be metabolic sinks for PAHs in the environment. Concentrations and toxicological implications of 1-hydroxypyrene, its derivatives, and analogous metabolites of other PAHs should be investigated.

Keywords: *Pyrene metabolism; cell-suspension and callus cultures of soybean, wheat, purple foxglove and jimsonweed; hydroxylation; O-methylation; carbohydrate conjugates*

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) found in the environment are mainly the product of incomplete combustion, and so, levels are preponderantly the result of anthropogenic activities (Edwards, 1983; Sims and Overcash, 1983). As environmental contaminants, PAHs are toxic (though little phytotoxic) and persistent, and many of them are mutagens and/or carcinogens (Sims and Overcash, 1983). Thus, the fate of PAHs in environmental compartments is of concern to both human health and ecotoxicology.

Plants are a main part of the human diet and the beginning of food chains. They make up most of the global biomass covering large areas of the Earth's land mass and are therefore especially exposed to PAH contaminations. Vegetation can act as a (metabolic) sink for xenobiotics (Nellessen and Fletcher, 1993; Sandermann, 1994). PAHs are detectable in air, soil, and plants (Edwards, 1983; Sims and Overcash, 1983; Jones et al., 1989; Simonich and Hites, 1994). In air, low molecular PAH compounds mainly exist in the vapor phase, whereas multiringed species predominate in the particulate fraction. Due to their lipophilic nature, PAHs are sorbed to soil organic matter, resulting in a decreased bioavailability; concentrations in plants are usually less than those in the soil where they grow (Edwards et al., 1982; Edwards, 1983; Sims and Overcash, 1983; Harms and Kottutz, 1990). Uptake of airborne PAHs by deposition on foliar plant parts was regarded as important. The compounds partition into the inert waxy cuticle and may be absorbed by the symplast and translocated (Edwards et al., 1982; Edwards, 1983; Ryan et al., 1989; Simonich and Hites, 1994).

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Though a number of papers deal with uptake and distribution, information on the plant metabolism of PAHs is scarce. Molecular structures of biotransformation products were only supposed or identified by cochromatography; partial support for metabolic pathways was derived from enzyme studies (Durmishidze et al., 1974; Edwards et al., 1982; Edwards, 1986; Harms, 1975, 1981, 1983, 1992; Harms and Kottutz, 1990; Harms and Langebartels, 1986; Harms et al., 1977; v.d. Trenck and Sandermann, 1978, 1980). Unequivocal data are not at hand.

Plant cell cultures provide defined systems for the study of xenobiotic metabolism. They are grown axenically and lack plant penetration and translocation barriers. Data obtained from metabolism studies are thus thought to represent the intrinsic enzymatic capacity of the plant species from which they are derived (Camper and McDonald, 1989; Komossa et al., 1995). Such studis, however, have to be validated with experiments using whole plants, to make sure that the same metabolites are formed in both systems. As a test system, heterotrophic cell suspensions of the dicot soybean and the monocot wheat were frequently employed for evaluation of the metabolic fate of xenobiotics in plants (Ebing et al., 1984; Harms and Langebartels, 1986; Komossa et al., 1995). However, crop plants were prevailingly investigated in metabolism experiments (Nellessen and Fletcher, 1993). Thus, with regard to comprehensive ecochemical evaluations of xenobiotics, we recently proposed to include wild plants in such screening procedures (v.d. Krol et al., 1995).

In this paper we describe, as an example, the metabolism of pyrene by plant cell cultures of soybean (*Glycine max*), wheat (*Triticum aestivum*), jimsonweed (*Datura stramonium*), and purple foxglove (*Digitalis purpurea*). In detail, the aims were to (1) evaluate the degree of biotransformation by different plant species using various culture conditions, (2) determine the

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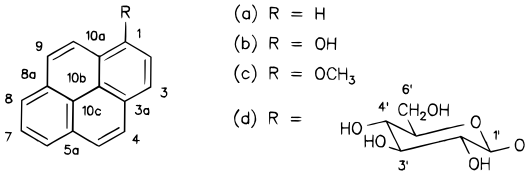

Figure 1. Chemical structures of (a) pyrene, (b) 1-hydroxypyrene, (c) 1-hydroxypyrene methyl ether, and (d) 1-(*O*-*β*-D-glucopyranosyl)pyrene.

percentages of soluble metabolites and nonextractable residues, and (3) isolate and identify the main metabolites. To our knowledge, we report here the first results on the metabolism of pyrene in plant tissues.

MATERIALS AND METHODS

Chemicals. [4,5,9,10-^{14}C]Pyrene (specific activity 1.195 GBq/mmol; radiochemical purity >99%; for chemical structures see Figure 1) was purchased from Sigma (Deisenhofen, Germany); unlabeled pyrene and 1-hydroxypyrene (1-HO-P) were supplied by Aldrich (Steinheim, Germany).

Synthesis of Reference Compounds. 1-(*O*-*β*-D-Glucopyranosyl)pyrene (Glc-*β*-1-HO-P) was synthesized from 1-hydroxypyrene and α-D-glucopyranosylbromide tetraacetate (Aldrich) according to the method of Ecke (1973), followed by deacetylation with LiOH. Its structure was confirmed by NMR spectroscopy [Varian VXR 300; in DMSO-d_6, ppm relative to Si(CH_3)$_4$, coupling constants J in hertz]: ^{1}H NMR (300 MHz) 8.56 [d, J = 9.0, H-C(10)], 8.25 [d, J = 8.7, H-C(3)], 8.23 [d, J = 7.7, H-C(8)], 8.22 [d, J = 8.1, H-C(6)], 8.15 [d, J = 9.4, H-C(9)], 8.11 [d, J = 9.1, H-C(5)], 8.04 [dd, J = 7.4, 7.7, H-C(7)], 8.03 [d, J = 8.7, H-C(4)], 7.92 [d, J = 8.7, H-C(2)], 5.21 [d, J = 7.7, H-C(1′)], 3.78 [d, J = 10.1, 2H-C(6′)], 3.55 [dd, J = 8.7, 11.4, H-C(3′)], 3.48 [m, H-C(5′)], 3.40 [dd, J = 8.7, 9.1, H-C(4′)], 3.29 [dd, J = 8.8, 9.0, H-C(2′)], assignments of signals at 8.23 and 8.22 may be inverted; ^{13}C NMR (75 MHz, proton decoupled) 151.29 [C(1)], 130.97 [C(5a)], 130.89 [C(8a)], 127.11 [C(10)], 126.32 [C(5), C(3)], 125.72 [C(4)], 125.54 [C(10b)], 125.16 [C(7)], 124.67 [C(10c)], 124.48 [C(8)], 124.26 [C(6)], 123.91 [C(3a)], 121.39 [C(9)], 120.06 [C(10a)], 113.14 [C(2)], 101.55 [C(1′)], 77.18 [C(5′)], 76.50 [C(3′)], 73.51 [C(2′)], 69.75 [C(4′)], 60.70 [C(6')], assignments of signals at 130.97 and 130.89 and at 124.48 and 124.26 may be inverted. Glc-*β*-1-HO-P was cleaved to 1-HO-P by *β*-glucosidase (see below) with 93% yield (HPLC; see below). Synthesis of 1-hydroxypyrene methyl ether (1-MeO-P) was performed by reaction of 1-HO-P with CH_3I in the presence of K_2CO_3 (Wildes et al., 1971). Structural confirmation was achieved by NMR spectroscopy (in $CDCl_3$): ^{1}H NMR 8.41 [d, J = 9.1, H-C(10)], 8.06 [d, J = 7.8, H-C(8)], 8.04 [d, J = 6.6, H-C(6)], 8.01 [d, J = 8.2, H-C(3)], 8.00 [d, J = 9.3, H-C(9)], 7.90 [dd, J = 7.1, 7.7, H-C(7)], 7.89 [d, J = 9.1, H-C(5)], 7.82 [d, J = 9.1, H-C(4)], 7.43 [d, J = 8.3, H-C(2)], 4.07 (s, OCH_3); assignments of signals at 8.06 and 8.04 may be inverted; ^{13}C NMR 153.63 [C(1)], 131.74 [C(5a)], 131.68 [C(8a)], 127.23 [C(10)], 126.35 [C(5)], 126.06 [C(3)], 125.77 [C(10b)], 125.44 [C(4)], 125.21 [C(10c), C(3a)], 124.93 [C(7)], 124.21 [C(8)], 124.12 [C(6)], 121.11 [C(9)], 120.19 [C(10a)], 108.01 [C(2)], 56.04 (OCH_3); assignments of signals at 124.21 and 124.12, at 126.35 and 126.06, and at 131.68 and 131.74 may be inverted. The electron impact MS spectrum (see below) was as follows: m/z (relative abundance) 232 $[M]^{\bullet+}$ (69), 217 (100), 190 (2), 189 (71), 188 (5), 187 (15), 94 (2).

Chromatographic Procedures. Analytical thin-layer chromatography (TLC) was performed on silica gel plates (SIL G-25; Macherey-Nagel, Düren, Germany) developed in solvent systems A1 [cyclohexane/dioxane (9:1 v/v)], A2 [*n*-hexane/diethyl ether (4:6 v/v)], A3 [*n*-hexane/diethyl ether/acetic acid (40:60:0.5 v/v/v)], A4 [*n*-hexane/toluene (9:1 v/v)], A5 (cyclohexane), A6 [*n*-hexane/toluene (92:8 v/v)], A7 [toluene/ethyl acetate/acetic acid (80:20:0.5 v/v/v)], B [ethyl acetate/2-propanol (4:6 v/v)], and C [ethyl acetate/2-propanol/water (65:24:12 v/v/v)]. ^{14}C zones were detected by means of a Tracemaster 40 radiochromatogram scanner (Berthold, Wildbad, Germany). Unlabeled reference compounds were located by fluorescence quenching (UV, 254 nm) and fluorescence at 366 nm. Preparative TLC was carried out on silica gel plates (SIL G-100).

High-performance liquid chromatography (HPLC) was performed with a Beckman System Gold Personal chromatograph (München, Germany) equipped with an ET 250/4 Nucleosil 100-5 C_{18} (5 °C using a flow rate of 0.8 mL min^{-1}). The column was connected to a Beckman Module 168 diode array detector (wavelength 254 nm, width 4 nm) and a Beckman 171 radioisotope detector. Elution was carried out with solvent system A[water/methanol (70:30 v/v)] for 1 min, followed by a linear 30 min gradient to water/methanol (5:95 v/v), isocratic water/methanol (5:95 v/v) for 9 min, and return to initial conditions within 1 min or solvent system B [same as A with 0.1% H_3PO_4 (v/v) replacing water]. All HPLC analyses were terminated by washing the column for 4 min with initial solvent.

GC/MS was performed on a HP5890 Series II gas chromatograph coupled to a HP5971A mass selective detector (Hewlett-Packard, Waldbronn, Germany). The system was equipped with an HP 5 column (cross-linked 5% Ph Me silicone; 50 m × 0.2 mm, 0.33 μm); temperature program: 100 °C for 2 min, 100− 280 °C at 15 °C min^{-1}, hold at 280 °C for 9 min; injector, 200 °C; interface, 300 °C; splitless injection. Electron impact MS spectra were recorded at 70 eV. Derivatization of 1-HO-P was performed with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA; Fluka, Neu-Ulm, Germany) at 70 °C for 30 min.

Plant Cell Cultures. Cell suspension cultures of soybean (*Glycine max* L. Merr. cv. Manadarin), wheat (*Triticum aestivum* L. cv. Heines Koga II), jimsonweed (*Datura stramonium* L.), and purple foxglove (*Digitalis purpurea* L.) were grown as described (Schmidt et al., 1993; v.d. Krol et al., 1995) and routinely subcultured every 7, 14, 9, and 9 days, respectively. Callus cultures were initiated by placing 0.5 g of suspension grown cells (wet weight) on medium (same as those of suspensions) solidified with 5 g L^{-1} Gel-Gro gelan gum (ICN, Meckenheim, Germany) and were cultivated at 27 °C in the dark. Scaled-up suspension cultures of wheat (to 2.5-fold size) were started by introducing 2.5 g of cells into 50 mL of medium (250 mL Erlenmeyer flasks).

Treatments and Incubation. Per assay, the cultures were treated with 20 μg (5 × 10^5 dpm in 20 μL of ethanol) or 50 μg of [4,5,9,10-^{14}C]pyrene (1.5 × 10^6 dpm in 50 μL of ethanol) in the scaled-up study. Jimsonweed and purple foxglove suspensions (five and four replicates, respectively) were subcultured and grown for 7 days. Then, [^{14}C]pyrene was applied, and the cultures were incubated for 48 h under conditions of routine cultivation. Fresh weights obtained after termination of experiments were 3.89 ± 0.28 and 1.97 ± 0.75 g, respectively (standard deviations at 95% confidence interval). Five days after subculturing, the suspensions of soybean (five replicates) were treated with [^{14}C]pyrene; 2 mL of 10% sucrose in water (w/v) was added, and the cultures were incubated for 96 h (4.36 ± 0.57 g fresh weight). Experiments with soybean and purple foxglove callus cultures (five replicates each) were started by placing an inoculum onto medium complemented with [^{14}C]pyrene and were incubated for 31 days (2.22 ± 0.70 g fresh weight) and 34 days (2.62 ± 1.76 g fresh weight), respectively. The wheat standard and scaled-up cell suspensions (seven and eight replicates, respectively) were treated with [^{14}C]pyrene directly after subculturing and were incubated for 14 days (2.41 ± 0.15 and 5.08 ± 0.23 g fresh weight, respectively). Inactivated control assays were performed with all experiments (two replicates each, except scaled-up study) using cells heated to 120 °C for 10 min prior to application.

Extraction and Determination of Distribution of ^{14}C. Suspension-cultured cells were separated from media by suction filtration and washed with water. Callus was removed with a spatula. The cells were placed in 40 mL of $CHCl_3$/methanol (1:2 v/v) according to the method of Bligh and Dyer (1959) in the cases of jimsonweed and purple foxglove suspensions or ethanol (other cultures) and were stored at −18 °C for 24 h. After extraction by means of sonication (Bandelin Sonoplus HD 200; Berlin, Germany), cell debris was separated and washed successively with respective extraction solvent and

Table 1. Percentage Distribution of ^{14}C after Incubation of Cell Cultures with 20 μg of [^{14}C]Pyrene[a]

cell culture (type, incubation period)	medium	cells			recovered ^{14}C
		cell extract	cyclohexane wash of cell debris	nonextractable residues	
soybean (suspension, 96 h)	19.4 ± 6.6	40.3 ± 19.4	56.6 ± 9.4	6.4 ± 2.7	122.7 ± 18.8
(callus, 31 days)	2.1 ± 0.4	30.0 ± 3.6	2.8 ± 0.7	20.8 ± 2.7	55.7 ± 4.9
wheat (suspension, 14 days)	3.5 ± 0.7	52.0 ± 9.5	2.4 ± 1.4	38.3 ± 3.4	96.2 ± 9.6
jimsonweed (suspension, 48 h)	1.8 ± 0.3	101.2 ± 6.4	3.9 ± 2.0	7.2 ± 0.9	114.1 ± 4.5
purple foxglove (suspension, 48 h)	7.4 ± 1.5	93.6 ± 4.6	1.3 ± 1.8	2.3 ± 0.5	104.6 ± 5.1
(callus, 34 days)	2.0 ± 0.4	18.6 ± 3.1	0.7 ± 0.5	25.3 ± 3.8	46.6 ± 2.4

[a] Average values ± standard deviations at the 95% confidence level are given.

cyclohexane (except scaled-up study); both organic fractions were handled separately. Insoluble cell debris was air-dried and combusted in a biological oxidizer OX 500 (Zinsser, Frankfurt, Germany) to evaluate portions of nonextractable residues. Soluble ^{14}C was quantitatively determined by liquid scintillation counting (LSC) using a Canbarra Packard Tricarb 1500 analyzer (Dreieich, Germany).

Aliquots of cell extracts were individually laid on TLC plates and developed successively with solvent systems A (A1, A2, A3, or A4, selected with regard to maximum separation of ^{14}C zones), B, and C. Cyclohexane phases and media of respective replicates were combined. Media were extracted with 1-butanol and concentrated; recoveries of ^{14}C in extracts were as follows: jimsonweed, 34%; purple foxglove, 42%; soybean, 70%; wheat, 29 %; wheat, scaled-up, 35%. 1-Butanol and cyclohexane fractions were also analyzed by TLC. In callus experiments, the ethanolic cell extracts were extracted with cyclohexane prior to further examination. Solid media were solubilized using 30 mL of 10 mM sodium citrate buffer (pH 6) at 40 °C for 30 min (Doner and Bécard, 1991) and extracted with ethyl acetate and 1-butanol. Extraction recoveries of ^{14}C were 49% (soybean) and 69% (purple foxglove).

Separation of Biotransformation Products. *Purple Foxglove Cell Suspensions.* Water was added to combined Bligh−Dyer extracts until phase separation was observed. The $CHCl_3$ layer was separated, and the aqueous layer was again extracted. After concentration, the chloroform phase (87% of ^{14}C found in cell extracts) was examined by TLC and subjected to multiple preparative TLC using solvent systems A5 and A1, consecutively.

Wheat Cell Suspensions. Combined cell extracts of standard experiments were concentrated. Remaining residues were suspended in water, extracted with 1-butanol, and analyzed by TLC. The radioactivity was separated by preparative TLC (systems A3 and B, consecutively) into three fractions (unchanged pyrene and metabolites mobile and immobile with system B). ^{14}C recovered in the latter two fractions amounted to 51% (in relation to 1-butanol extract) and was further separated (successively TLC systems A1, B, and C) into four and three fractions, respectively. The fractions were analyzed by TLC and subjected to enzymatic and acidic hydrolysis (see below).

In the case of the scaled-up experiment, the combined ethanolic cell extracts were extracted with cyclohexane (removal of unchanged pyrene; 7% of ^{14}C in cell extracts), concentrated, and extracted with 1-butanol as described. Remaining aqueous (18%) and 1-butanol phases (67%) were examined by TLC (solvent systems A3, B, and C, consecutively); ^{14}C was separated as described.

Hydrolysis Procedures. Enzymic cleavage (ca. 5×10^4 dpm each) was performed with 1 mg of almond β-glucosidase (EC 3.2.2.21; Sigma) in 4 mL of 0.1 M Na_2HPO_4/0.05 M citric acid buffer (pH 4.4). After incubation (6 h, 37 °C), the mixture was extracted with diethl ether and 1-butanol. Hydrolysis was also executed with 2 M HCl by refluxing at 100 °C for 2 h, and the reaction mixture was similarly extracted.

RESULTS

Distribution of Radioactivity and Turnover of Pyrene. After termination of incubation, the radioactivity detected in the experiments was mainly associated with the cells (Table 1). In the soybean suspensions, 19.4% of applied ^{14}C was found in the media, whereas corresponding values were below 8% in the other cultures (3.3% in wheat scaled-up suspension). With the exception of soybean cell suspensions, soluble radioactivity in the cells was recovered almost quantitatively by extraction with chloroform/methanol or ethanol, whereas minor amounts were removed by an additional cyclohexane wash from cell debris. In the wheat cells, 38.3 and 29.0% (scaled-up assays) were found as bound, nonextractable residues that remained associated with cell debris after extraction. Portions below 10% were detected in the other suspensions, and higher amounts emerged in callus cultures. Thus, about 100% of applied ^{14}C was recovered in the assays with cell suspension cultures, verifying that pyrene was not volatilized, degraded to volatile organic compounds, or mineralized. In the assays performed on solid media, recoveries amounted to about 50%. Remaining ^{14}C portions, however, could be extracted from cotton wool plugs used to close the culture flasks. Due to TLC, these consisted entirely of pyrene that had obviously evaporated from the systems.

In inactivated suspension cultures, <5% of applied ^{14}C was found in media and nonextractable residues each (11% in soybean media). The remaining radioactivity was detected in cell extracts. With soybean and wheat, 42 and 14%, respectively, were removed by washing cell debris with cyclohexane. The controls of soybean and purple foxglove callus cultures, respectively, revealed 6 and 3% in the media 9 and 1% as bound residues. In the former, 10% of applied ^{14}C was in cyclohexane.

The amounts of ^{14}C in media were low. In addition, pyrene almost quantitatively volatilized, when laid on TLC plates as aqueous solution; no volatilization was observed with organic solvents. Thus, replicates of media were combined and extracted with 1-butanol. Subsequently, 1-butanol extracts, cell extracts, and cyclohexane fractions were analyzed by TLC (solvent system A) to determine portions of unmetabolized pyrene. Radioactive zones were found that cochromatographed with nonlabeled pyrene ($R_f = 0.40-0.59$) and at the origin. At this stage of the investigation, the latter were regarded as pyrene transformation products. Media and cyclohexane fractions prevailingly contained pyrene, while ^{14}C at $R_f = 0.00$ was mainly found in cell

extracts. Large amounts of metabolites (50−70 %) emerged in wheat. The results from the TLC analysis of cell extracts and extracts of media together with the respective bound residue fractions shown in Table 1 suggested that the degree of pyrene biotransformation—defined as the percentage of soluble metabolites plus that of nonextractable residues (Ebing et al., 1984; Harms and Langebartels, 1986; Komossa et al., 1995)—was low in dicot suspensions (15−22%), intermediate in callus cultures (30−37%), and high in the monocot wheat (88−100%). In the control assays, turnover was generally smaller (1−15%).

The individual extracts were further examined by TLC using solvent systems A, B, and C, consecutively. Unexpectedly, in the assays with soybean and jimsonweed, the amounts of radioactivity regarded as pyrene metabolites ($R_f = 0.00$) were considerably lower in more polar solvents. Concomitantly, the ^{14}C zone assigned to the parent increased; no further zones emerged. Since 1-HO-P and pyrene were separated ($R_f = 0.71$ and $R_F = 0.77$, respectively, with system B), cochromatography of parent with metabolites was excluded. It was thus supposed that pyrene exhibited strong interference during TLC separation. This interpretation was supported by the fact that, repeatedly, significant portions of pyrene appeared during fruitless attempts to isolate presumed "polar products" from jimsonweed assays. Analogous observations were previously published on PAHs (v.d. Trenck and Sandermann, 1978; Edwards et al., 1982). Extracts derived from soybean callus cultures were additionally analyzed by HPLC (system A). Each of the respective chromatograms revealed only one ^{14}C peak ($R_t = 35.9$ min) cochromatographing with authentic pyrene. With regard to quantity and quality (correlation coefficients of UV spectra, 0.99), ^{14}C of samples was positively identified with pyrene. We arrived at the conclusion that in soybean and jimsonweed cultures, soluble metabolites of pyrene were present only in trace amounts—if at all.

Separation and Identification of Biotransformation Products. *Purple Foxglove Cell Suspensions.* The combined cell extracts were separated into aqueous and chloroform phase. TLC analysis of the latter using solvent systems A1 (and A5) showed three radioactive zones: 3% of ^{14}C on the plate at $R_f = 0.00$ ($R_f = 0.00$), 8% at $R_f = 0.34$ ($R_f = 0.13$), and 89% at $R_f = 0.46$ ($R_f = 0.31$; cochromatographing with pyrene). The metabolite detected at $R_f = 0.34$ (6.5% of applied ^{14}C) was isolated by preparative TLC. Concerning polarity, the compound ranged between parent and 1-HO-P: pyrene $R_f = 0.44$, 0.39, and 0.31, 1-HO-P $R_f = 0.10$, 0.05, and 0.00, metabolite $R_f = 0.34$, 0.18, and 0.13 using systems A1, A4, and A5, respectively, and cochromatographed with synthesized 1-MeO-P ($R_f = 0.38$, 0.17, and 0.14). The sample dissolved in methanol/water (1:1 v/v) was examined by HPLC (system A). While pyrene, 1-HO-P, and 1-MeO-P, respectively, had $R_t = 36.0$, 30.1, and 36.95 min, respectively, the metabolite eluted at $R_t = 36.7−37.0$ min. The UV spectrum of the transformation product resembled those of 1-HO-P and 1-MeO-P (correlation coefficients of 0.73 and 0.90, respectively) but differed from that of the parent. UV spectra of authentic 1-HO-P and 1-MeO-P were correlated (0.99). In experiments with callus cultures, the same metabolite (3.9% of applied ^{14}C) was also detected besides unidentified polar material (8.7%; $R_f = 0.00$). We concluded that the main metabolite formed by purple foxglove was the methyl ether of 1-hydroxypyrene.

Wheat Cell Suspensions. The 1-butanol phase (52.0% of applied ^{14}C) derived from combined cell extracts was analyzed by TLC (successively solvent systems A3, B, and C). Besides pyrene (10% of ^{14}C present on plate), the chromatogram recorded after system C demonstrated seven radioactive zones with R_f values smaller than that of the parent (listed with increasing polarity: 2, 5, 30, 5, 19, 21, and 9%). The radioactivity was roughly separated by preparative TLC into (i) unchanged pyrene, (ii) metabolites mobile, and (iii) metabolites immobile with system B. Further separations of (ii) and (iii) afforded moderate polar fractions M-1 (system C, $R_f = 0.74$), M-2 (0.38), M-3 (0.37), M-4 (0.32), and highly polar M-5 (0.21), M-6 (0.19), and M-7 (origin), respectively, which had radiochemical purities between 82 and 100%. Minor product M-1 was only obtained as a mixure (2:8) with the main metabolite M-3 and cochromatographed with synthesized Glc-β-1-HO-P ($R_f = 0.50$ using system B).

The fractions were separately incubated with β-glucosidase, and organic extracts of reaction mixtures (73−100% recovery of ^{14}C) were analyzed by TLC. All metabolites were transformed, thus proving that they were sugar-conjugated biotransformation products of pyrene; however, except M-1, none was quantitatively cleaved to one single ^{14}C product. The aglycon released from M-1 cochromatographed with 1-HO-P. Though amounts isolated were too low for unequivocal identification, it was assumed that metabolite M-1 was the β-glucoside of 1-HO-P. ^{14}C zones cochromatographing with 1-HO-P were also liberated from all other fractions; however, portions were below 20%. Presumably, metabolites M-2 to M-7 were disaccharides and oligosaccharides metabolically processed from Glc-β-1-HO-P (M-1).

We arrived at the same conclusion after HCl treatment. Only 1-HO-P was released from every fraction (29−92% of ^{14}C) and identified by TLC (A3, $R_f = 0.40$; successively A3 and B, $R_f = 0.71$; A7, $R_f = 0.51$), HPLC (system B; $R_t = 29.9$ min; correlation coefficients of UV spectra >0.90), and GC/MS ($R_t = 17.50− 20.14$ min) as trimethylsilyl derivative. The mass spectra were identical to that of authentic 1-HO-P trimethylsilyl ether: *m/z* (relative abundance) 290 $[M]^{\bullet+}$ (100), 275 (18), 259 (12), 244 (7), 217 (6), 201 (9), 189 (12), 137 (4), 100 (1), 73 (26), 45 (9).

From the wheat scaled-up assays, a similar metabolite pattern was obtained. However, the metabolism of pyrene was shifted to more polar transformation products, as compared to the preceding experiment. Metabolite M-1 was absent; the main product was again M-3 (23% of applied ^{14}C). Preparative TLC yielded radiochemically pure M-3 ($R_f = 0.43$ after system C), which by treatment with HCl liberated 1-HO-P (identified by HPLC and TLC). During HPLC analysis (system A), metabolite M-3 exhibited $R_t = 20.82$ min; the correlation coefficient of its UV spectrum with that of 1-HO-P was 0.97. Authentic Glc-β-1-HO-P and 1-HO-P were observed at $R_t = 23.69$ and 30.31 min, respectively.

DISCUSSION

A low vapor pressure was reported for pyrene (9.12×10^{-5} Pa at 20 °C; Sims and Overcash, 1983); Henry's constant (2.8×10^{-1}; Ryan et al., 1989), however, indicates that the compound may volatilize. Similar observations were reported on PAHs (Knorr, 1966; Edwards et al., 1982; Edwards, 1986; Jones et al., 1992). Volatilization of pyrene observed in the present study

in assays performed with callus cultures agrees with these data. Pyrene is remarkably lipophilic (log $K_{O/W}$ = 5.32; 140 μg L^{-1} aqueous solubility; Sims and Overcash, 1983); the quantity applied in the experiments was above solubility. Thus, pyrene had a high affinity for the plant tissues and was rapidly absorbed, as substantiated by the distributions of ^{14}C in both inactivated and native cultures. Data reported on three- and five-ringed PAHs using cell suspension cultures of mono- and dicots (Harms 1975, 1981, 1983, 1992; Harms and Kottutz, 1990; Harms and Langebartels, 1986; Harms et al., 1977; v.d. Trenck and Sandermann, 1978) led to the supposition that absorption depended on molecular size, structure, and plant species: three-ringed PAHs were easily absorbed, and differences emerged with five-ringed species. In the present study, four-ringed pyrene behaved like three-ringed PAHs. The exceptional distribution of the compound found in soybean cell suspensions remained inexplicable.

Generally, the plant metabolism of xenobiotic compounds is subdivided into three phases. Simple biotransformations (e.g. oxidation, hydrolysis) of exocon molecules are termed phase I, while phase II comprises conjugation reactions of the parent or phase I metabolites with endogenous substances such as carbohydrates, glutathione, or amino acids. Phase III processes are characterized by compartmentation and storage. Export into the apoplast may lead to adsorptive and covalent integration of exocon moieties into cell wall macromolecules (e.g. lignin, cellulose, pectin), resulting in nonextractable residues (Lamoureux and Rusness, 1986; Sandermann, 1994; Komossa et al., 1995).

In the present study, nonextractable residues were detected in all investigated species and were thus regarded as the main route of the fate of pyrene in the cell cultures. Differences in amounts of the phase III products between native and heat-inactivated assays indicated that their formation largely required viable cells. The nonextractable fraction also increased with time as shown with callus cultures. However, the chemical nature of the pyrene-derived bound residues remains unclear. This is especially true of soybean and jimsonweed, for which soluble phase I and II metabolites were not detected. It is questionable whether bound residues resulted from covalent linkages or noncovalent inclusion into plant macromolecules.

Due to strong interference during TLC, the degree of turnover of pyrene could be determined only with difficulty. The screening procedure mentioned earlier (Ebing et al., 1984; Harms and Langebartels, 1986; Komossa et al., 1995) turned out to be insufficient and was supplemented by additional extraction and HPLC analysis. Accordingly, the turnover of pyrene in jimsonweed and soybean suspensions and in callus cultures of soybean amounted to 7.2, 6.4, and 20.8% of applied ^{14}C, respectively, resulting only from nonextractable residues. In purple foxglove suspensions and callus cultures, standard and scaled-up suspensions of wheat, 8.8, 37.9, 86.1, and 94.0%, respectively were found transformed to both soluble metabolites and bound residues. Investigations published on PAH metabolism in plant tissues agree that the extent of transformation depends on the PAH compound and the plant species. In general, lower molecular weight two- and three-ringed PAHs (naphthalene, anthracene, phenanthrene) should be biodegraded more rapidly than higher molecular weight five-ringed compounds (e.g. perylen, benzo[*a*]pyrene, dibenz[*a*,*h*]anthracene); angular may be considered more stable than linear species (Cerniglia, 1992; Sims and Overcash, 1983). Using suspension cultures, noticeably high degrees of transformation were reported on benzo[*a*]pyrene (73%) and perylen (61%) in soybean (soluble metabolites, nonextractable residues), whereas corresponding values from wheat were 38 and 10%, respectively (Harms and Langebartels, 1986; Komossa et al., 1995). Published results (Harms and Kottutz, 1990) showed that anthracene was metabolized in high degree (soybean, 59%; wheat, 68%) and phenanthrene turnover was lower (soybean, 36%; wheat, 20%). It was also reported (Harms, 1983) that perylen, benzo[*a*]pyrene, and dibenz[*a*,*h*]anthracene exhibited low turnover (<20%) in cell cultures of four *Chenopodiaceae* species, with the exception of benzo[*a*]pyrene in *C. rubrum* (about 70%). The present data largely agree with both the general estimations and results of previous metabolism studies. Degradability of pyrene by the plant tissues roughly ranged between three-ringed and five-ringed PAHs and was extremely dependent on plant species. Concerning the data published on turnover of benzo[*a*]pyrene in soybean and *C. rubrum* suspensions (Harms, 1983; Harms and Langebartels, 1986), discrepancies with our results remain, unless extremely plant species specific metabolic capacities are considered. Judged from our experiments, the high turnover reported on benzo[*a*]pyrene may have resulted from TLC artifacts and insufficient extraction of cell debris.

The main metabolite identified in purple foxglove cultures, 1-hydroxypyrene methyl ether, was supposed to be derived from 1-HO-P by methylation. Though methyl group transfer to phenolic substrates is common with natural plant constituents (Harborne, 1979), O-methylation of xenobiotic compounds or their metabolites is limited and may be regarded as unusual transformation to lipophilic conjugates (phase II). So, metabolism studies using lupine cell cultures, soybean, and spinach plants (Langebartels and Harms, 1985; Casterline et al., 1985) demonstrated that pentachlorophenol (PCP) and metabolites were O-methylated besides carbohydrate conjugation. We could demonstrate that purple foxglove suspensions converted 4-nitrophenol to its β-glucoside (unpublished data). Thus, possible explanations for formation of 1-MeO-P are, first, exogenously applied phenols may be channeled differently from those emerging intracellularly by metabolism. Second, distribution within plant cells of highly lipophilic phenols as PCP (log $K_{O/W}$ = 5.01; Ryan et al., 1989) and 1-HO-P (exhibiting R_f values similar to those of PCP) possibly differs from that of less lipophilc compounds, e.g. 4-nitrophenol (log $K_{O/W}$ = 1.91; Ryan et al., 1989), entailing different accessibilities to crucial enzyme activities.

In wheat supensions, pyrene was also converted first to phase I product 1-HO-P, which, however, was then conjugated with sugar moieties. Higher plants have a noticeable capacity to biotransform (xenobiotic) phenolic substrates to carbohydrate conjugates; most frequently, these are glucosides. Monosaccharides may be processed by addition of further sugar molecules (Harborne, 1979; Komossa et al., 1995; Lamoureux and Rusness, 1986; Sandermann, 1994; Schmidt et al., 1993; v.d. Krol et al., 1995). In the present study with wheat cell cultures, Glc-β-1-HO-P (M-1) was detected only as a minor product. The main metabolite M-3 was shown to be a carbohydrate conjugate of 1-HO-P, and due to chromatographic behavior may be a disaccharide possibly derived from M-1. Further, more polar conjugates

of 1-HO-P detected presumably were oligosaccharides. Though di- and oligosaccharides of xenobiotics were reported, the great number of conjugates observed in wheat was unexpected. In contrast to plants, the metabolism of pyrene was investigated in the fungus *Cunninghamella elegans* (Cerniglia et al., 1986), and results resembled those obtained with wheat cell suspensions. The compound was found hydroxylated by the *C. elegans* at the 1-, 1,6-, and 1,8-positions with subsequent glucosylation.

The first step of pyrene metabolism was hydroxylation at position 1 in wheat and purple foxglove. Data on the metabolism of benzo[*a*]pyrene in plant tissues indicated that the PAH was transformed to hydroxylated derivatives (Durmishidze et al., 1974; Harms, 1975, 1983; Harms et al., 1977); structural proof was lacking. Quinones of benzo[*a*]pyrene identified by v.d. Trenck and Sandermann (1980) were supposed to arise by (abiotic) autoxidation of a hydroxylated derivative. Thus, the present unequivocal identification of 1-HO-P as a primary phase I product of pyrene supports previously published suppositions. In the plant metabolism of endogenous and exogenous compounds, hydroxylations of aromatic rings are common and may be catalyzed by cytochrome P-450 dependent systems, peroxidases, or hydroperoxidases (Dohn and Krieger, 1981; Cole, 1983; Sandermann, 1994). Oxidation of benzo[*a*]pyrene was demonstrated with microsomal fractions from pea and soybean (v.d. Trenck and Sandermann, 1980). The transformation was NADPH independent in contrast to reactions catalyzed by cytochrome P-450 systems. Involvement of peroxidases in the formation of 1-HO-P is unlikely due to pyrene's high ionization potential (7.50 eV; Rogan et al., 1980). A hydroperoxidase system (Dohn and Krieger, 1981; Cole, 1983) would easily explain 1-hydroxylation of pyrene; catalysis by the system was reported to be independent of NADPH and required hydroperoxides of fatty acids as sources of oxidizing power.

Two conclusions may be drawn from the present study, provided that enzymatic capacities found in cell cultures are qualitatively the same as those of intact plants: with regard to ecochemical evaluations, plants are—dependent on species—a metabolic sink for pyrene and possibly PAHs in general; both crop and wild plants should be integrated in screening procedures. In the human diet, 1-HO-P, its derivatives, and analogous metabolites of other PAHs may be present and accumulate; their toxicological implications on human health should be investigated.

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