Degradation of Aromatic Compounds in Plants Grown under Aseptic Conditions

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The aim of the work is to investigate the ability of higher plants to absorb and detoxify environmental pollutants – aromatic compounds via aromatic ring cleavage. Transformation of ^{14}C specifically labelled benzene derivatives, [1–6-^{14}C]-nitrobenzene, [1–6-^{14}C]-aniline, [1-^{14}C]- and [7-^{14}C]-benzoic acid, in axenic seedlings of maize (*Zea mays* L.), kidney bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.) and pumpkin (*Cucurbita pepo* L.) were studied. After penetration in plants, the above xenobiotics are transformed by oxidative or reductive reactions, conjugation with cell endogenous compounds, and binding to biopolymers. The initial stage of oxidative degradation consists in hydroxylation reactions. The aromatic ring can then be cleaved and degraded into organic acids of the Krebs cycle. Ring cleavage is accompanied by $^{14}CO_2$ evolution. Aromatic ring cleavage in plants has thus been demonstrated for different xenobiotics carrying different substitutions on their benzene ring. Conjugation with low molecular peptides is the main pathway of aromatic xenobiotics detoxification. Peptide conjugates are formed both by the initial xenobiotics (except nitrobenzene) and by intermediate transformation products. The chemical nature of the radioactive fragment and the amino acid composition of peptides participating in conjugation were identified.

Key words: Benzene Derivatives, Degradation, Plants

Introduction

Benzene derivatives are ubiquitous environmental pollutants. Their fate in the environment and their sensitivity to degradation systems in plants are determined by the nature of the substituents carried by their aromatic ring. Earlier studies have shown that plants, like microorganisms (Gottschalk, 1979), are able to degrade benzene by several pathways including oxidative cleavage of the aromatic ring and incorporation of the resulting products in the cell regular metabolism (Durmishidze and Ugrekhelidze, 1969; Barz *et al*., 1978).

Ever since numerous literary data prove uptake and mineralization of benzene or its derivatives by higher plants (Ferro *et al*., 1997; Ugrekhelidze *et al*., 1997; see for review Korte *et al*., 2000). However, evidence for ring cleavage of aromatic compounds in plants is scarce. Besides the question of the actual capacity of plants to perform this type of reaction, some reasons could explain that observation: ring-labelled molecules are not always available and experiments are often aimed at identifying xenobiotic metabolites rather than estimating the emitted CO_2 and the accompanying low molecular weight degradation products.

In the present work, we have tried to clear up some existing ambiguity about the ability of plants to degrade aromatic rings. With this aim in view, we have investigated the transformation of [1–6-^{14}C]-nitrobenzene, [1–6-^{14}C]-aniline, and [1-^{14}C]- and [7-^{14}C]- (*i.e.* carboxyl-^{14}C) benzoic acid in annual plant seedlings grown under aseptic conditions.

Materials and Methods

Transformation of nitrobenzene, aniline and benzoic acid was studied in 7- to 10-day-old seedlings of maize (*Zea mays* L.), kidney bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.) and pumpkin (*Cucurbita pepo* L.). Plant seeds were supplied from Natakhtari Experimental Station, Georgia.

[1–6-^{14}C]-Nitrobenzene was synthesized by [1–6-^{14}C]-benzene nitration with nitric acid (Becker *et al*., 1976), and muconic acid by phenol oxidation with acetic acid hydroperoxide (MacDonald and Stainer, 1957). Radiochemical purity of the la-

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belled compounds was checked by chromatography and autoradiography.

[1–6-^{14}C]-Aniline, [1-^{14}C]- and [7-^{14}C]-benzoic acid were commercially obtained (Isotop, St. Petersburg, Russia).

In order to grow seedlings under aseptic conditions, maize and kidney bean seeds were soaked in concentrated sulfuric acid (1 min), then in a 1% solution of mercury chloride (maize seeds, 15 min; kidney bean seeds, 5 min). Pea and pumpkin seeds were soaked in concentrated sulfuric acid for 2–3 min, then in a 15% solution of hydrogen peroxide (15–20 min), and finally in 1% mercury chloride (pea seeds, 10 min; pumpkin seeds; 15 min). After each treatment, the seeds were washed with sterile distilled water. Germination was carried out on sterile moist quartz sand at 26–27 °C. Germinated seeds were transferred into vessels of special construction containing sterile Knop nutrient medium and provided with a sterile air supply and a carbon dioxide trap.

After 7–10 d of growth, the nutrient medium was replaced by aqueous solutions of [1–6-^{14}C]-nitrobenzene (concentration 1.5×10^{-2} mol/l, specific radioactivity 2.02×10^{7} Bq/g), [1–6-^{14}C]-aniline (concentration 1.5×10^{-2} mol/l, specific radioactivity 1.85×10^{7} Bq/g), [1-^{14}C]-benzoic acid (concentration 8×10^{-4} mol/l, specific radioactivity 3.75×10^{6} Bq/g) or [7-^{14}C]-benzoic acid (concentration 1.64×10^{-3} mol/l, specific radioactivity 6.2×10^{7} Bq/g). At the end of incubation (after 72 h at 25–27 °C) the leaves and roots were harvested and weighed. They were fixed in 80% boiling ethanol, then homogenized in the same solvent. The homogenates were separated into a soluble fraction (tissue extract) containing low molecular weight compounds and an insoluble fraction (non-extractable residues), mainly comprising biopolymers. The radioactive carbon dioxide produced by the plants was absorbed in a trap containing a monoethanolamine-methylcellosolve mixture (9:1) (Ugrekhelidze *et al*., 1999).

The soluble fraction was concentrated under vacuum at 40 °C and separated according to Shkolnik and Doman (1961) into three subfractions: organic acids, amino acids and sugars. After separation, the labelled compounds were analyzed by autoradiography (Schütte, 1966) and visualized by colour reactions: carbonic acids by bromophenol blue or bromocresol blue, sugars by anilino phtalate or ammonium molybdate, amino acids by ninhydrin, and phenolic compounds by diazotized sulphanilic acid (Ugrekhelidze *et al*., 1999).

Radioactive compounds not identified by colour reactions were further purified by two-dimensional chromatography in the solvent systems ethanol/formic acid/water (16:1:3 v/v/v) and butanol/formic acid/water (6:1:5) and hydrolyzed in boiling 6 N hydrochloric acid during 12 h. After several extractions of hydrolyzates with diethyl ether, the resulting ether and water solutions were concentrated under vacuum and analyzed by autoradiographic, chromatographic and spectroscopic methods, as well as by colour reactions.

The tissue extracts containing benzoic acid transformation products were divided into two parts. One was submitted to steam distillation. The distillate was acidified with hydrochloric acid, saturated with sodium chloride and extracted by diethyl ether. After concentration, radioactive phenol could be identified in the ether extract. To improve the precision of the radioactivity measurements, non-radioactive phenol was added to the other part of the tissue extract. After steam distillation as above, bromine water was added to the distillate. The white precipitate was recrystallized from ethanol/water, then dissolved in 5% sodium sulphite and reprecipitated with concentrated hydrochloric acid.

Radioactivity was measured with a Rack Beta II liquid Scintillation counter in Brag's hydrophilic system (Rapkin, 1970).

Results and Discussion

Distribution of radioactivity

The distribution of radioactivity after absorption of labelled nitrobenzene and aniline showed that the molecules and/or their metabolites were absorbed through the roots and translocated to the aerial parts of the seedlings. After 72 h of metabolism, the major part of the radioactivity was present in the soluble fraction (tissue extract). The insoluble (unextractable) residues contained one third or less of the absorbed radioactivity. Moreover, all incubations of axenic seedlings with ring-labelled nitrobenzene and aniline, and also with benzoic acid, always resulted in some evolution of $^{14}CO_2$, showing that plant tissues were able to degrade the aromatic xenobiotics. However, if one refers to the amounts of radioactive CO_2 produced, the extent of degradation differed significantly between the various molecules. It was

shown that nitrobenzene was appreciably degraded (3.8 to 11.4% of the total assimilated radioactivity), while aniline degradation was rather low (0.3 to 1.0%). By contrast, the degradation of either [1-^{14}C]- or [7-^{14}C]-benzoic acids was comparatively high and varied from 11 to 25% depending on plant species. In case of [7-^{14}C]-benzoic acid, the emitted CO_2 probably mostly resulted from the decarboxylation of the molecule. Evidence for active decarboxylation of benzoic acid was obtained in experiments with isolated pea chloroplasts (Mithaishvili *et al.*, 1979).

Primary metabolites

The universal character of the hydroxylation of hydrophobic xenobiotics in plants was demonstrated by the *o*-hydroxylation of nitrobenzene. After 72 h of metabolism, even trace amounts of untransformed nitrobenzene could not be found in the plants analyzed in the experiments. The main metabolite isolated and identified from acid hydrolyzates was *o*-nitrophenol. Trace amounts of *p*-nitrophenol were also found in the same hydrolyzates.

Intermediate metabolites of nitrobenzene were then searched after a shorter incubation, in which pea seedlings were allowed to absorb [1–6-^{14}C]-nitrobenzene for 18 h. On these conditions, *o*-nitrophenol, the primary product of nitrobenzene oxidative transformation, was found in the free and in conjugated form. The overall amount of nitrophenol (free plus conjugated) amounted to 23.1% of the total radioactivity in roots. In leaves, by contrast, *o*-nitrophenol was less abundant (5.4%), and the main metabolite was aniline, which accounted for 30.3% of the total radioactivity.

Like nitrobenzene, [1–6-^{14}C]-aniline was oxidized to low molecular weight compounds. In our experiments, *o*-aminophenol, the primary product of aniline oxidation, was isolated and identified from ether extracts of acid hydrolyzates. Identification was carried out according to a colour reaction with $FeCl_3$ and UV spectroscopy in ethanol at 235 nm. The presence of *o*-aminophenol among the aniline transformation products indicates that a notable part of the absorbed aniline undergoes *ortho*-hydroxylation.

In contrast to the above observations, the detection of the primary metabolites of ring-labelled benzoic acid was more elusive. Although the initial stage of aromatic ring oxidative degradation is hydroxylation, the high reactivity of hydroxylated metabolites usually results in their quick disappearance from the cellular medium, so that their isolation is not always possible. For that reason, we were not able to identify hydroxybenzoic acid or its conjugated forms, which are commonly formed in higher plants (Cooper-Driver *et al.*, 1972; Terashima *et al.*, 1976). As a matter of fact, the detection of radioactive phenol in the present experiments showed that benzoic acid must be successively hydroxylated and decarboxylated. It can be supposed that the velocities of the two processes are equal or that decarboxylation is faster. In both cases, identification of hydroxybenzoic acid is practically impossible.

The above results show that plants grown under aseptic conditions are able to metabolize aromatic xenobiotics to hydroxylated derivatives. However, the formation of *o*-nitrophenol during nitrobenzene hydroxylation was rather unexpected. The orientation of hydroxylation in mono-substituted benzene is determined by the chemical nature of the pre-existing substituent. Electrophilic substituents direct hydroxylate mainly to the *ortho* and *para* positions. In animals, phenol is transformed into pyrocatechol and hydroquinone (Capel *et al.*, 1972; Kaufman, 1976), and aniline into *o*- and *p*-aminophenol (Parke, 1960). By contrast, the nitro group is a very strong deactivator of the aromatic ring. Under its influence, the new group introduced by an electrophilic substitution is mainly oriented to the *meta* position. In biological systems however, that order of preferences can be modified, and in addition to the *meta* position, the substitutions are oriented to the *para* position. In rabbits for example, nitrobenzene is mainly transformed into *m*- and *p*-nitrophenols, the *ortho*-isomer being found in trace amounts (Parke, 1956; Beauchamp *et al.*, 1982). The orientation of substitution reactions can also be modified in plants. For example, the carboxylic group of benzoic acid directs hydroxylation to the *meta* position. In the plant *Gaultheria prokumbeas* however, *o*- and *p*-hydroxybenzoic acids are formed instead of *m*-hydroxybenzoic acid (El-Basuiuni *et al.*, 1964).

Finally, it should be also emphasized that besides oxidation reactions, plants are able to perform reductions. Transformation of nitrobenzene to aniline is notable in pea roots, and is the most prominent reaction in leaves.

Ring cleavage and resulting metabolites

In general, after 72 h of metabolism of the three xenobiotics, a small part (3–4%) of the total radioactivity was found incorporated into amino acids, sugars and organic acids. Incorporation of ^{14}C in amino acids and sugars was very low, however. A higher radioactivity was associated with the fraction containing the organic acids, especially with succinic, fumaric and muconic acids (Tables I, II). With the production of radioactive CO_2, the formation of these open-chain acids proves the splitting of the aromatic rings of nitrobenzene, aniline and benzoic acid.

Aromatic ring cleavage is preceded by the formation of the *o*-dihydroxyarenic intermediate, then the ring is cleaved mainly between the hydroxyl groups. In animals and microorganisms, the degradation of benzene results in the formation of muconic acid (Gottschalk, 1979). In the same way, the oxidation and decarboxylation of benzoic acid by microorganisms leads to pyrocatechol, and further oxidative degradation leads to the formation of muconic acid (Yoshikawa *et al.*, 1990; Gibson, 1993). The results of Table I show that in higher plants, the transformation of benzoic acid led to the formation of open-chain compounds, muconic acid and ensuing metabolites, with phenol and pyrocatechol as intermediate products. According to the results of Table II, the degradation of nitrobenzene and aniline proceeded via the same pathway. These results agree with previous data showing that in plants, the primary product of phenol ring oxidative degradation is muconic acid, formed by pyrocatechol ring cleavage (Ugrekhelidze *et al.*, 1999). Muconic acid β-oxidation then leads to fumaric acid. The formation of other Krebs cycle tricarboxylic acids indicates that in plants, the carbon skeleton of xenobiotic aromatic rings can be de-

Table I. Incorporation of [1-^{14}C]-benzoic acid label into organic acids fraction[a].

Plant	Radioactivity (%)						
	Muconic acid	Fumaric acid	Succinic acid	Malic acid	Glycolic acid	Glyoxylic acid	Malonic acid
Maize	24.5	18.5	9.0	12.5	11.5	13.0	11.0
Pumpkin	33.0	24.0	12.0	–	16.0	15.0	–
Pea (root)	33.0	22.0	10.0	–	11.0	10.0	14.0

[a] Concentration 8×10^{-4} mol/l, specific radioactivity 3.75×10^6 Bq/g; exposure time 72 h; 25–27 °C.

Table II. Incorporation of [1–6-^{14}C]-nitrobenzene and [1–6-^{14}C]-aniline radioactive label into the organic acids fractions[a].

Plant		Radioactivity (%)								
		Fumaric acid	Succinic acid	Malic acid	Glycolic acid	Glyoxylic acid	Malonic acid	Oxalic acid	Citric acid	X[b]
Maize	Roots	26.5 (75.8)	21.6 (14.6)	8.5 (6.0)	– (–)	21.4 (–)	11.3 (–)	2.0 (–)	– (–)	8.7 3.6
	Leaves	27.0 (24.0)	14.2 (–)	16.0 (23.9)	10.2 (–)	14.2 (23.9)	11.5 (–)	4.2 (–)	– (16.7)	2.7 (11.5)
Kidney bean	Roots	17.4 (72.3)	20.1 (–)	15.1 (1.6)	– (–)	16.0 (10.0)	14.0 (11.3)	12.5 (–)	– (–)	4.9 (4.8)
	Leaves	20.2 (41.4)	12.1 (9.2)	15.6 (8.6)	14 (–)	12.7 (27.3)	12.3 (–)	7.9 (–)	– (–)	5.2 (13.5)
Pea	Roots	21.8 (85.4)	16.3 (–)	15.2 (5.7)	19.8 (–)	9.3 (–)	14.7 (7.8)	– (–)	– (–)	2.9 (1.1)
	Leaves	16.1 (40.3)	21.4 (21.0)	17.4 (1.4)	5.1 (–)	17.2 (–)	9.1 (29.9)	3.2 (–)	– (7.4)	10.5 (–)

[a] Concentration of labelled nitrobenzene 1.5×10^{-2} mol/l, specific radioactivity 2.0×10^7 Bq/g; concentration of labelled aniline 1.5×10^{-2} mol/l, specific radioactivity 1.85×10^7 Bq/g; exposure time 72 h; 25–27 °C. Data for [1–6-^{14}C]-aniline is given in parenthesis.
[b] Unidentified organic acids.

graded and that individual carbon atoms can be introduced into the general metabolism via fumaric acid.

According to the above data, the utilization by plants of the elements of aromatic xenobiotics for their own metabolism was especially intensive in the case of [1-^{14}C]-benzoic acid. The transformation and utilization of nitrobenzene and aniline aromatic rings was carried out with lower intensity.

Two main conclusions can be drawn from the above results:

1) Aromatic compounds can be degraded in plants via ring splitting.
2) The intensity of the aromatic ring oxidative degradation significantly depends on the nature of the substituents as well as the plant species.

Formation of conjugates

According to the existing data, most xenobiotics undergo conjugation with endogenous compounds after their entry in plant cells. Depending on its chemical properties, a xenobiotic can be conjugated either in an unchanged form, or, more usually, after transformation into a partially oxidized metabolite. As a result, in most cases, toxicity and other physiological activities are greatly reduced.

In the present experiments, low molecular metabolites were analyzed after acid hydrolysis of the soluble extracts. The identified metabolites of nitrobenzene were products of reduction or hydroxylation reactions – aniline, *o*-nitrophenol, and *p*-nitrophenol in trace amount. Thus, as it can be logically expected, nitrobenzene must be converted into intermediate products in order to form conjugates.

Hydrolyzates of aniline metabolites were divided in a radioactive, ether-soluble fraction, and a non-radioactive, water-soluble fraction, as explained in Materials and Methods. Analyses allowed identifying these metabolites as conjugates of aniline and *o*-aminophenol with peptides. The data of Table III show that conjugates of various chromatographic mobilities differed by their amino acid composition. Moreover, negative test reactions showed that the phenolic and primary amino groups of aniline metabolites were involved in the formation of peptide conjugates. Thus the initial xenobiotic and its transformation intermediate *o*-aminophenol are both substrates for conjugation with peptides.

Table III. Amino acid composition of peptide conjugates of [1–6-^{14}C]-aniline metabolites[a].

Plant	Conjugate R_f	Amino acid composition of peptide conjugates
Maize		
Roots	0.82	Asp, Glu, Ala, Met
Leaves	0.95	Asp, Glu, Ala, Met, Ser, Trp
	0.97	Asp, Glu, Ala, Ser, Met, Trp, Leu
	0.96	Asp, Glu, Ser, Gly, Tyr, Leu
	0.98	Asp, Ser, Gly, Tyr, Val, Leu
Kidney bean		
Roots	0.85	Asp, Glu, Ser, Leu
Leaves	0.90	Asp, Glu, Trp, Ala, Ser
	0.97	Asp, Glu, Trp, Ala, Ser
	0.92	Asp, Glu, Gly, Ala, Leu, Met
	0.96	Asp, Glu, Gly, Ala, Leu, Met
Pea		
Roots	0.84	Asp, Glu, Gly
Leaves	0.97	Asp, Glu, Gly, Ala
	0.75	Asp, Glu, Ser, Trp
	0.83	Asp, Glu, Ser, Tyr, Val, Leu
	0.93	Asp, Glu, Ser, Tyr, Val, Leu

[a] Solvent system: butanol/formic acid/water (6:1:5 v/v/v); chromatographic paper FN-6 ("Filtrak", Germany).

Analysis of conjugates of benzoic acid revealed that they all contained benzoic acid as a radioactive fragment. The non-radioactive components of acid hydrolyzates were peptides of different amino acid composition. As peptide conjugates did not give positive test reactions of carboxylic and primary amino groups, it is concluded that the carboxylic group of benzoic acid was linked to the primary amino groups of peptides. The solubility and chromatographic mobility of the conjugates show that they derive from low molecular peptides. In the plants studied here, benzoic acid conjugates were mainly formed in roots, their amount being low in leaves.

Unextractable residues

A sizeable portion of the radioactivity (15.9 to 32.2% for nitrobenzene, 5.3 to 33.3% for aniline) was incorporated in insoluble residues, very likely composed of biopolymers. Acid hydrolyzates of the labelled biopolymers did not contain the initial xenobiotics or some of their transformation products, suggesting that ester or amide bonds were not involved. One possibility is that, during metabolization, quinones resulting from the dehydration of dihydroxyarenic intermediates react with

protein sulfhydryl and amino groups, and irreversibly bind to them. Another possibility is that the xenobiotics and/or their metabolites are covalently incorporated into lignin networks.

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