Metabolism of Benzoic Acids and Phenols in Cell Suspension Cultures of Soybean and Mung Bean

Wolfgang Barz, Rita Schlepphorst, and Peter Wilhelm

Lehrstuhl für Biochemie der Pflanzen der Universität, Münster/Westfalen

and

Karl Kratzl and Erich Tengler

Institut für Organische Chemie der Universität, Wien/Oesterreich

Z. Naturforsch. **33 c**, 363–367 (1978); received April 12, 1978

Benzoic Acids, Phenols, Ring Fission, Peroxidases, Polymerisation, Plant Cell Suspension Cultures

Uptake of benzoic and salicylic acid by soybean cell suspension cultures is diffusion-mediated and exclusively leads to glucose-esters. — Veratric acid is *para*-demethylated to vanillic acid which is conjugated to a monoglucoside. — Nitrobenzoic acids are scarcely taken up by cell cultures with glucose-esters as sole products. — Ring-labelled derivatives of vanillylalcohol and creosol are mainly polymerized to insoluble structures, ring cleavage reactions could not be observed.

Introduction

Previous studies [1–7] on benzoic acid metabolism in plants and plant cell suspension cultures have shown the occurrence of several alternative metabolic pathways such as oxidative polymerisation, conjugation with carbohydrates, aspartic or malic acid, *para*-O-demethylation, ring-fission of *ortho*-dihydroxy compounds and oxidative decarboxylation of *para*-hydroxy substituted acids. The quantitative ratio between polymerisation, glucosylation and degradation for a particular compound is of interest in view of the possible application of plant cell cultures for biotechnological purposes [8] and for the understanding of the capacity of plant cell cultures to meabolize unnatural organic compounds (*i. e.* xenobiotics) [9].

We now describe experiments which focus on quantitative aspects of uptake and conjugation of simple benzoic acids, of nitro-substituted acids and on degradation and polymerisation of various ring-labelled compounds.

Experimental

Materials

[Carboxyl-^{14}C]benzoic acid (spec. radioact. 41.6 mCi/mmol) and [carboxyl-^{14}C]salicylic acid (spec. radioact. 31.1 mCi/mmol) were obtained from the Radiochemical Centre, Amersham. [Carboxyl-^{14}C]-*p*-hydroxybenzoic acid (spec. radioact. 0.07 mCi/mmol) and [3-O-methyl-^{14}C]veratric acid (spec. radioact. 0.25 mCi/mmol) were available from earlier studies [3]. [Carboxyl-^{14}C]-*m*-nitrobenzoic acid was obtained by nitration (0.3 ml conc. HNO_3, 0.35 ml conc. H_2SO_4) of [carboxyl-^{14}C]benzoic acid (10 mg) at room temperature for 3 hours. After addition of water (10 ml) and $NaHCO_3$ (pH 2) the solution was extracted with ether and the product was obtained by TLC in systems S_4 and S_5. Yield 66 percent, spec. radioact. 0.08 mCi/mmol. [Carboxyl-^{14}C]-*m*-nitrobenzoic acid methylester was prepared by reaction of the acid with diazomethan in methanol and the product purified in S_5 and S_7. [Carboxyl-^{14}C]-3-nitro, 4-hydroxybenzoic acid was obtained from [carboxyl-^{14}C]-4-hydroxylbenzoic acid (30.6 mg) according to [10]. The product was isolated by TLC in S_4 (R_F 0.38) and repeatedly rechromatographed (S_5, S_4). Yield 60%, spec. act. 0.03 mCi/mmol.

Syntheses of [3-^{14}C]-5-nitrosalicylic acid (**II**) (spec. radioact. 0.06 mCi/mmol) and [5-^{14}C]vanillin (**V**) (spec. radioact. 0.048 mCi/mmol) have been published [18, 19]. [5-^{14}C]vanillylalcohol (**VI**) was prepared from **V** (0.2 mmol) in acetonitrile (5 ml) by reduction with 0.24 mmol $NaBH_4$ for 20 minutes under reflux. The solvent was evaporated and the residue taken up in dil. HCl. The product was extracted with ether and purified by TLC in system S_8, spec. act. 0.048 mCi/mmol yield 0.18 mmol (90%), m. p. 113–115 °C (ref. [20]

Request for reprints should be sent to Prof. Dr. W. Barz, Lehrstuhl für Biochemie der Pflanzen, Hindenburgplatz 55, D-4400 Münster.

114 – 115 °C). [6-^{14}C]-2-methoxy-4-methyl-phenol (**VII**) was prepared from **V** (1 mmol) by heating (140/150 °C) and stirring for 5 hours in triethylamine (3 ml) with hydrazine hydrate (250 mg) and powdered KOH (1.5 g) under oxygen-free nitrogen. The reaction mixture was poured on ice, acidified with dil. HCl and extracted with CH_2Cl_2. After removal of solvent the product was purified by distillation. Yield 0.95 mmol (95%).

For the preparation of [1-^{14}C]-2-hydroxy-3-methoxy-5-methylbenzaldehyde (**VIII**) 0.725 mmol **VII** was treated with 2.15 mmol freshly sublimed hexamethylenetetramine in 2 ml 50% acetic acid under reflux for 1.5 hours. After the addition of 0.5 ml 6 N HCl reflux was continued for another 30 minutes, 40 ml water were added and the mixture was steam-distilled. The product was isolated from the distillate with ether and purified by TLC in S_8. Yield 0.43 mmol (59%), m.p. 77 °C (ref. [21] 77 °C). [1-^{14}C]-2-hydroxy-3-methoxy-5-methylbenzylalcohol (**IX**) was obtained from **VIII** (0.2 mmol) by $NaBH_4$-reduction as described above for **VI**. Purification of the product by TLC in S_8 [yield 0.18 mmol (90%), m.p. 51 – 53 °C (ref. [22] 53 °C)].

Cell cultures and feeding experiments

Cultivation of soybean and mungbean cell suspension cultures, application of substrates, collection of $^{14}CO_2$, fractionation of cells, measurement of soluble and insoluble material for radioactivity and determination of radioactivity on chromatograms was accomplished as previously described [3, 6]. Uptake of substrate was followed by decrease of radioactivity in the medium.

Chromatography

The following systems were used for PC on Whatman 3 MM paper:

S_1: *n*-butanol : acetic acid : water = 4 : 1 : 1,
S_2: water,
S_3: *n*-butanol : water : methanol = 40 : 19 : 11,

TLC on silicagel was carried out with

S_4: dibutyl ether : *n*-hexane : acetic acid = 80 : 16 : 4,
S_5: benzene : dioxane : acetic acid = 40 : 25 : 4,
S_6: ethylacetate : pyridin : water = 4 : 1 : 1,
S_7: methanol : acetic acid = 9 : 1,
S_8: benzene : acetic acid : water = 4 : 2 : 1.

Hydrolyses

The purified conjugates were hydrolysed a) with H_2SO_4 (2 N) at 100 °C for 5 hours; for sugar determination excess sulfuric acid was removed by precipitation with $Ba(OH)_2$, b) with trifluoroacetic acid at 50 °C for up to 24 hours; the product was obtained by evaporation of the acid, c) with hog-liver esterase (E.C. 3.1.1.1.) (Boehringer, spec. act. 100 U/mg protein) in sodiumphosphate buffer, pH 7.5, d) with β-glucosidase (E.C. 3.2.1.21 [Serva Heidelberg] and the same conditions as c). In test assays the esterase was shown to hydrolyse *p*-methoxybenzoic acid glucoseester quantitatively but proved to be completely inactive towards *p*-nitrophenol-β-glucoside.

Quantitative determinations

Glucose was quantitated by using the glucose-oxidase/peroxidase standard procedure (Boehringer). Liberated aglycones were determined spectrophotometrically using appropriate standard curves.

Results

Experiments with benzoic acids

Earlier studies [11] have shown that uptake and metabolism of benzoic and cinnamic acids may depend greatly on the substrate concentration in the nutrient medium and that conjugation may be linked to uptake [8]. A quantitative investigation of [carboxyl-^{14}C]benzoic acid and [carboxyl-^{14}C]salicylic acid uptake was started to measure both the efficiency of acid uptake and the cellular capacity for conjugating external acids. In parallel sets of experiments (0.15 μCi/flask) the two acids were added to soybean cell suspension cultures in concentrations between 10^{-5} to 10^{-3} M. Even the highest concentrations were quantitatively absorbed within 24 hours, salicylic acid, however, was taken up somewhat slower than benzoate. Fig. 1 indicates that uptake of the two acids is obviously by diffusion because no indication of any saturable uptake system could be obtained. Analyses of cellular extracts after 24 hours of incubation revealed that the cells still contained appreciable amounts (app. 3 – 29%) of free benzoic acids. It required more than 48 hours at 10^{-3} M external concentration before the absorbed benzoic acids were quantitatively converted to conjugates. $^{14}CO_2$ was not formed in these experiments.

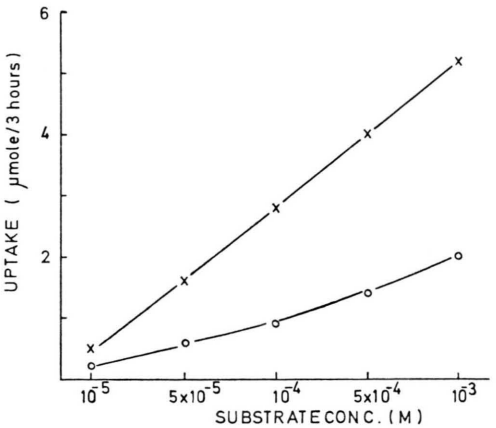

Fig. 1. Concentration related uptake of [carboxyl-^{14}C]benzoic acid (×—×) and [carboxyl-^{14}C]salicylic acid (○—○) in soybean cell suspension cultures. Fresh weight of cells was identical within one experiment.

Investigations of the conjugated material (TLC in S_5, PC in S_1, S_2 and S_3 followed by acid hydrolysis) led only to unchanged benzoic and salicylic acid, respectively. The glucose esters were identified as main metabolites; structural proof for salicylic acid glucose ester was obtained by quantitative hydrolysis with a liver esterase which does not attack phenolic glucosides.

In previous studies [3] exogenously fed veratric acid (3,4-dimethoxybenzoic acid) was strongly *para*-demethylated and stored as a vanillic acid conjugate while similarly applied vanillic acid was predominantly decarboxylated. Further investigations on this compartmentalized benzoic acid metabolism required structural elucidation of the vanillic acid conjugate formed. Thus, [3-O-methyl-^{14}C] veratric acid (10^{-4} M) was added to soybean cell suspension cultures for 36 hours and cell extracts were worked up for vanillic acid conjugates. Only one compound of this type could be isolated (purification in S_1, S_2, S_3; UV spectrum λ_{max} MeOH 251, 288 nm, λ_{min} 235, 274 nm) which contained 1 mol glucose as sole conjugating partner. Treatment of the conjugate with esterase failed to hydrolyse the compound, but after methylation of the conjugate with diazomethan and hydrolysis with trifluoroacetic acid vanillic acid methyl ester (TLC comparison with veratric acid in S_4, S_5) was obtained as the only radioactive product. Therefore, vanillic acid formed by *para*-O-demethylation of veratric acid is transformed to vanillic acid-O-glucoside and thus protected against oxidative decarboxylation [6].

Experiments with nitrobenzoic acids

Metabolism of nitro substituted aromatic compounds has scarcely been investigated in plants [12, 13] though compounds of this type (*i. e.* trifluralin) are claimed to be degraded by plant systems [14]. To further explore the capacity of plant cell suspension cultures for metabolising foreign compounds, experiments with [carboxyl-^{14}C]-*m*-nitro benzoic acid (**I**), [3-^{14}C]-5-nitrosalicylic acid (**II**), [carboxyl-^{14}C]-3-nitro, 4-hydroxybenzoic acid (**III**) and [carboxyl-^{14}C]-*m*-nitrobenzoic acid methyl ester (**IV**) were conducted. Decarboxylation, formation of amino compounds by reduction of nitro-groups [15], oxidative polymerisation reactions and conjugation of absorbed substrates were of special interest in these studies. Cell suspension cultures of soybean and mungbean were used and incubated with **I** to **IV** in concentrations of 10^{-3} M to 5×10^{-5} M for up to 24 hours. In comparison to the above mentioned benzoic acids the uptake of nitrobenzoic acids was severely reduced, soybean cell suspension cultures even totally failed to absorb any amount of **I** or **II** at either concentration, while **III** entered the cells to only 40%. Mungbean cell cultures showed values for uptake of 100% for **III**, 65% for **I** and 12% for **II** at 10^{-4} M during 6 hours. Application of **IV** instead of **I** to soybean cell suspension cultures did not significantly increase the uptake of the *m*-nitrobenzoic acid skeleton.

In all studies with **I** to **IV** no $^{14}CO_2$ was formed by the two cell cultures nor was any radioactivity incorporated into ethanol insoluble cell residues. Chromatographic analyses of cell extracts (S_1, S_3, S_5, S_7) revealed that practically no free unconjugated substrates could be discovered. Investigations on the purified conjugates of **I** and **III** by hydrolysis with esterase and β-glucosidase and quantitative determinations of aglycone and sugar demonstrated for both compounds the exclusive formation of glucose esters.

Experiments with ring-labelled substrates

Due to the rather limited number of available ring-labelled aromatics, evidence for ring-cleavage reactions of aromatic compounds in plants is scarce and mainly restricted to a few *ortho-* [2, 3, 16] and *para-* [17] dihydroxy-compounds or derivatives yielding such structures by demethylation or hydroxilation.

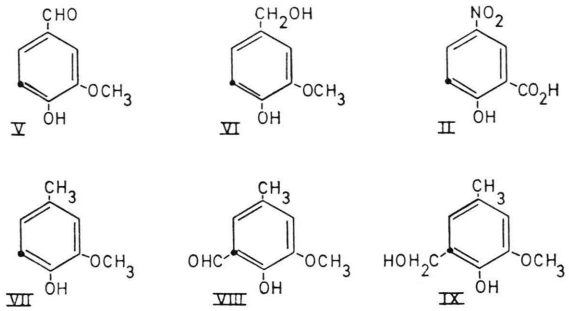

Fig. 2. Structures and position of label (•) of compounds investigated in cell cultures. **II** has been investigated in the section of nitrobenzoic acids.

The ring-labelled compounds shown in Fig. 2 were synthesized (see Experimental) in continuation of earlier studies [18, 19] on position-specific ring-labelled compounds and applied (10^{-4} M/24 – 72 h) to cell cultures of soybean and mungbean with the intention to measure ring-fission reactions versus polymerisation and conjugation. In this respect, compounds **VII** to **IX** are of special interest because they may either yield *ortho*-dihydroxy derivatives suitable for ring-cleavage reactions or form good substrates for polymerisation by phenolase-peroxidase hydroxilation. The distribution of label according to established fractionation schemes showed the high sensitivity of these substrates towards oxidative processes. While the uptake of all substrates proceeded very well (>90%), the high rate of incorporation of label (>50%) into insoluble cell structures (polymers) and ether-insoluble, water soluble material (oligomers) [8, 11], documents the importance of polymerisation reactions in phenol metabolism. Ring fission occurred if at all to an insignificant degree only (max. $^{14}CO_2$-values 0.2%). Furthermore, no unchanged substrate nor any conjugates of unchanged substrates could be found as indicated by chromatographic analyses of the ether extracts before and after hydrolysis according to method b). The bulk of radioactivity located in the fraction of polar water-soluble compounds which tend to polymerize on TLC and have so far resisted chemical elucidation [6] was gradually incorporated into insoluble cell structures.

Short term incubations with cell cultures and control experiments with horseradish peroxidase indicated that the peroxidase-catalyzed oxidation and polymerisation [6] of compounds such as **V**, **VI**, **VIII**, and **IX** are likely the predominant reactions occuring in cell cultures.

Discussion

Exogenous application of various aromatic compounds to plant cell cultures mainly resulted in conjugation and polymerisation reactions. With respect to the formation of glucose esters for which the cells have a very high capacity our data on salicylic acid and 3-nitro-4-hydroxy-benzoic acid are in accord with various studies [24, 25] in plants but differ partly from work in *Datura innoxia* suspension cultures [4] and other plants [26] where glucose esters and mono-glucosides of *para*- and *ortho*-hydroxybenzoic acid have both been found. Mono-glucoside formation in our cultures seems to be restricted to vanillic acid which stems from *para*-demethylation of veratric acid. Addition of glucose to the phenolic hydroxy group of this vanillic acid explains the surprising lack of any decarboxylation reaction which readily occurs upon exogenous feeding [2, 3]. The previously postulated compartmentalisation of benzoic acid metabolism [3] now finds further support in the observation of different conjugates. Studies on the specificity and localisation of the relevant glucosyltransferases would be helpful in explaining the observed differences. Such enzymes must not be expected to be associated with uptake processes as postulated [8, 25] for conjugation reactions and as shown by the high levels of free acids in the cells. The subsequent metabolic fate of the formed conjugates upon further growth of the cell cultures as well as the repeatedly made observation of mixtures of conjugates [25] in plants can be elucidated by further studies with aromatic acids in plant cell cultures while hydroxilation reactions (*i. e.* salicylic to gentisic acid) [26] are occurring only to an insignificant extent.

Our previous [3] and present data and those by the Japanese workers [4] further differ with respect to polymerisation of phenols. Except for salicylic acid and 3-nitro-4-hydroxybenzoic acid all phenols investigated (see Fig. 2 and earlier publications [3, 6, 8]) were strongly incorporated into polymeric material. This plant-characteristic detoxication mechanism [7] seems to be largely due to peroxidases which abundantly occur in cell cultures [6].

In view of our data it therefore seems surprising that hydroquinone, catechol or *p*-hydroxy-benzoic acid are claimed to be quantitatively converted to glucosides [4]. Obviously the *D. innoxia* cell cul-

ture differs strikingly from all cultures investigated so far for polyphenol metabolism. Isolation of cell lines low in peroxidase content therefore seems appropriate for further studies on polyphenol metabolism.

Studies on nitro substituted aromatics in our plant cell cultures were severely hampered by the very slow uptake. The reduced capacity in cellular uptake in the sequence **III** > **I** > **II** and be correlated with a decrease in pK-values from 4.02 (**III**), 3.44 (**I**) to 2.9 (**II**) supporting the assumption that optimum uptake is observed when the pH of the medium and the pK of the substrate are similar. One can further say that due to the nitro group the 3-nitro-4-hydroxy-benzoic acid (**III**) was not decarboxylated as previously shown to occur with *p*-hydroxybenzoic acids [3] due to peroxidases in the medium.

The present findings may further be summarized by saying that *in vivo* studies with plant cell cultures are of very limited use with those substrates (**V** to **IX**) which are preferentially polymerized by peroxidases as now known from a wide variety of other phenols [27].

Support by Deutsche Forschungsgemeinschaft, Fond zur Förderung der wiss. Forschung (Oesterreich) and Fonds der Chemischen Industrie is gratefully acknowledged.

[1] H. Harms, H. Söchtig, and K. Haider, Z. Pflanzenphysiol. **64,** 437 (1971).
[2] J. Berlin, W. Barz, H. Harms, and K. Haider, FEBS Lett. **16,** 141 (1971).
[3] H. Harms, K. Haider, J. Berlin, P. Kiss, and W. Barz, Planta **105,** 342 (1972).
[4] M. Tabata, F. Ikeda, N. Hiraoka, and M. Konoshima, Phytochemistry **15,** 1225 (1976).
[5] P. S. Juneja and R. K. Gholson, Phytochemistry **15,** 647 (1976).
[6] J. Berlin and W. Barz, Z. Naturforsch. **30 c,** 650 (1975).
[7] B. E. Ellis, Lloydia **37,** 168 (1974).
[8] W. Barz, Plant Cell Cultures and Its Bio-technological Application (W. Barz, E. Reinhard, and M. H. Zenk, eds.), pp. 153 ff, Springer-Verlag, Berlin, Heidelberg, New York 1977.
[9] H. Sandermann, H. Diesperger, and D. Scheel, reference 8, pp. 178 ff.
[10] H. Roberts, J. Chem. Soc. London **196,** 200 (1935).
[11] W. Barz, Planta Medica, Supplement 117 (1975).
[12] J. B. Pridham and M. J. Saltmarsch, Biochem. J. **87,** 218 (1963).
[13] A. Goris, P. Frigot, D. Molho, J. Aknin, P. Muller, and M. Cibault, Phytochemistry **10,** 679 (1971).
[14] G. W. Probst, T. Golab, R. J. Herberg, F. J. Holzer, F. J. Parka, C. van der Schaus, and J. B. Tepe, J. Agric. Food Chem. **15,** 592 (1967).
[15] K. Kieslich, Microbiol Transformations of non-steroid cyclic compounds, pp. 127 ff, G. Thieme Publishers, Stuttgart 1976.
[16] B. E. Ellis, FEBS-Letters **18,** 228 (1971).
[17] R. Durand and M. H. Zenk, FEBS-Letters **39,** 218 (1974).
[18] K. Kratzl, F. W. Vierhapper, and E. Tengler, Mh. Chem. **106,** 321 (1975).
[19] K. Kratzl and F. W. Vierhapper, Mh. Chem. **102,** 224 (1971).
[20] J. A. Pearl, J. Org. Chem. **12,** 79 (1947).
[21] R. H. F. Manske and A. E. Ledingham, Can. J. Res. **22 B,** 115 (1944).
[22] E. Ziegler and K. Mayer, Mh. Chem. **92,** 246 (1961).
[23] J. Berlin and W. Barz, Planta **98,** 300 (1971).
[24] M. H. Zenk, Z. Naturforsch. **19 b,** 398 (1964).
[25] G. H. N. Towers, Biochemistry of Phenolic Compounds (J. B. Harborne, ed.), pp. 249 ff., Academic Press, London, New York 1964.
[26] G. Copper-Driver, J. J. Corner-Zamodits, and T. Swain, Z. Naturforsch. **27 b,** 943 (1972).
[27] C. Steelink, Rec. Adv. Phytochem. **4,** 239 (1972).