DEGRADATION OF PHENOLIC COMPOUNDS IN PLANT CELL CULTURES

J. BERLIN and W. BARZ

Lehrstuhl für Biochemie der Pflanzen, Biologisches Institut II der Universität Freiburg/Br., Germany

and

H. HARMS and K. HAIDER

Institut für Biochemie des Bodens, Forschungsanstalt für Landwirtschaft Braunschweig-Völkenrode, Germany

Received 8 June 1971

1. Introduction

Secondary aromatic plant constituents have recently been shown to be subject to catabolic reactions [1–4]. For cinnamic and benzoic acids turnover has also been demonstrated i.e. elimination of two carbons atoms of the side chain with the first [2, 5] and oxidative decarboxylation with the latter [6–8] yielding various substituted phenols. Furthermore, demethylation of methoxylated cinnamic acids has been observed [2, 9–11] together with a low percentage of ring-fission reactions [10] for both benzoic and cinnamic acids.

Demonstration of catabolic reactions and elucidation of their pathways in higher plants are severely hampered by the necessity to work with aseptically grown plants [7, 8, 11]; low concentrations of the metabolites formed as well as extensive binding of the phenolic substrates to ethanol insoluble polymeric structures [8, 12]. Use of plant cell suspension cultures can be expected to circumvent some of the difficulties described. The rapid degradation of 2′,4,4′,6′-tetrahydroxy chalcone to *p*-coumaric acid and *p*-hydroxybenzoic acid respectively [13] as well as the notable formation of $^{14}CO_2$ from phenylpropanoid compounds [14] and ring-labelled aromatic amino acids [15] by various plant cell cultures provide evidence for this assumption.

We report the results of studies designed to show degradation of various ^{14}C-labelled benzoic and cinnamic acids. Cell suspension cultures of mungbean and soybean extensively and rapidly produced $^{14}CO_2$ from such substrates labelled in carboxyl groups, methoxyl groups or ring carbon atoms. As measured by $^{14}CO_2$ production some of the reactions nearly went to completion indicating the superiority for such studies of cell cultures over intact plants.

2. Experimental

2.1. *Methods*

Cell suspension cultures of mungbean (*Phaseolus aureus Roxb.*) and soybean (*Glycine max*) were grown as described in previous communications [14, 16]. The cultures of mungbean were used on the 12th day and those of soybean on the 7th day after inoculation. The labelled phenolic acids were dissolved in growth medium and either autoclaved (15 min, 121°) or sterilized by filtration. 5 ml of substrate solution were pipetted into the cell suspensions (40 ml in 200 ml Erlenmeyer flasks) giving final concentrations ranging from 10^{-4} M to

Table 1
Percent of radioactivity evolved as $^{14}CO_2$ from labelled catechol, benzoic acids and cinnamic acids by cell suspension cultures of mungbean.

Compound added	Percent of total radioactivity in CO_2 (hr)						Sum of total radioactivity liberated as CO_2 (%)
	3	6	9	24	48	72	
(A)							
(1) Catechol-(U-^{14}C)	–	–	–	0.17	0.15	–	0.32
(B) Benzoic acids							
1. *Carboxyl*-^{14}C							
(2) Benzoic acid	2.1	0.7	0.87	0.53	0.64	0.25	5.09
(3) Salicylic acid	0.2	< 0.1	< 0.1	0.4	0.4	1.75	ca. 2.95
(4) *p*-Hydroxybenzoic acid	9.2	1.6	0.2	1.0	0.5	1.1	13.6
(5) Protocatechuic acid	7.7	3.9	1.2	2.2	4.4	1.9	21.3
(6) Vanillic acid	36.3	1.0	0.2	1.2	4.5	3.8	47.0
(7) Syringic acid	26.9	13.6	0.7	1.5	2.7	0.3	45.7
2. *Methyl*-^{14}C							
(8) Vanillic acid	–	–	–	1.4	0.7	0.6	2.7
(9) Syringic acid	–	–	–	1.4	2.2	0.2	3.8
(10) 3,4,5-Trimethoxybenzoic acid	–	–	–	15.5	1.4	1.0	17.9
3. *Ring* ^{14}C							
(11) Benzoic acid	–	–	–	< 0.1	< 0.1	< 0.1	ca. 0.3
(12) *p*-Hydroxybenzoic acid	–	–	–	0.07	0.05	0.05	0.17
(13) Anisic acid	–	–	–	0.00	0.00	0.00	0.00
(14) Protocatechuic acid	–	–	–	5.0	2.4	2.0	9.4
(15) Vanillic acid	–	–	–	0.12	0.2	0.1	0.42
(C) Cinnamic acids							
1. *C-3*-^{14}C							
(16) Cinnamic acid	–	0.2	–	0.35	0.27	0.19	1.01
(17) *p*-Coumaric acid	–	0.65	–	0.9	0.48	0.51	2.54
2. *Methyl*-^{14}C							
(18) *p*-Methoxycinnamic acid	–	2.9	–	13.7	9.5	3.5	29.6
3. *Ring* ^{14}C							
(19) Caffeic acid	–	–	–	2.9	0.74	0.7	4.34

5×10^{-4} M (0.3–1.0 μCi). The Erlenmeyer flasks were placed on a New Brunswick Gyrotory shaker (120 rpm) and in the dark a gentle stream of sterile air passed through the suspensions. $^{14}CO_2$ was collected in a mixture of ethylene glycol monomethylether/ethanolamine (2:1, v/v) [17]. Suitable aliquots of the absorbent solution were counted for radioactivity in a Beckman LS 100 scintillation counter and counting efficiencies determined by internal standardization (toluene-^{14}C). All manipulations with the cell cultures were performed under strictly aseptic conditions and sterility of the cell suspensions controlled after each experiment by streaking aliquots of the solutions on standard nutrient agar I (Merck). The petri dishes were kept at 30° for 5 days.

Table 2
Percent of radioactivity evolved as $^{14}CO_2$ from labelled catechol, benzoic acids and cinnamic acids by cell suspension cultures of soybean.

Compound added	Percent of total radioactivity in CO_2 (hr)						Sum of total radioactivity liberated as CO_2 (%)
	3	6	9	24	48	72	
(A)							
(1) Catechol-(U-^{14}C)	0.17	0.47	–	2.55	1.32	–	4.51
(B) Benzoic acids							
1. *Carboxyl-^{14}C*							
(2) Benzoic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(3) Salicylic acid	–	0.1	0.1	0.38	0.52	0.32	1.42
(4) *p*-Hydroxybenzoic acid	8.5	8.8	1.7	1.0	0.4	0.7	21.1
(5) Protocatechuic acid	37.2	6.75	3.5	10.1	5.8	2.6	65.95
(6) Vanillic acid	75.1	2.9	1.0	2.1	1.3	0.6	83.0
(7) Syringic acid	75.4	8.2	1.2	2.0	1.0	0.5	88.3
2. *Methyl-^{14}C*							
(8) Vanillic acid	–	–	–	0.6	0.5	0.4	1.5
(9) Syringic acid	–	–	–	4.5	5.8	4.4	14.7
(10) 3,4,5-Trimethoxy-benzoic acid	–	–	–	4.2	6.0	4.5	14.7
3. *Ring ^{14}C*							
(11) Benzoic acid	–	–	–	0.0	0.0	0.0	0.0
(12) Anisic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(13) *p*-Hydroxybenzoic acid	–	–	–	0.68	0.23	0.2	1.01
(14) Protocatechuic acid	–	–	–	7.1	3.5	1.9	12.5
(15) Vanillic acid	–	–	–	0.2	0.17	0.15	0.42
(C) Cinnamic acids							
1. *C-3-^{14}C*							
(16) Cinnamic acid	–	–	–	1.64	0.62	0.21	2.47
(17) *p*-Coumaric acid	–	–	–	1.72	1.06	0.44	3.22
2. *Methyl-^{14}C*							
(18) *p*- Methoxycinnamic acid	–	1.42	1.08	6.4	7.1	4.9	20.9
3. *Ring ^{14}C*							
(19) Caffeic acid	–	–	–	3.82	1.45	0.95	6.22

2.2. *Materials*

Benzoic acid-(ring-^{14}C), -(carboxyl-^{14}C), salicylic acid-(carboxyl-^{14}C) and cinnamic acid-(3-^{14}C) were purchased from the Radiochemical Center Amersham and Departement des Radioelements, Gif-sur-Yvette, respectively. The syntheses and purification of all other labelled compounds have been described [9, 18, 19]. The ring labelled compounds used were uniformly labelled in each carbon atom of the aromatic nucleus.

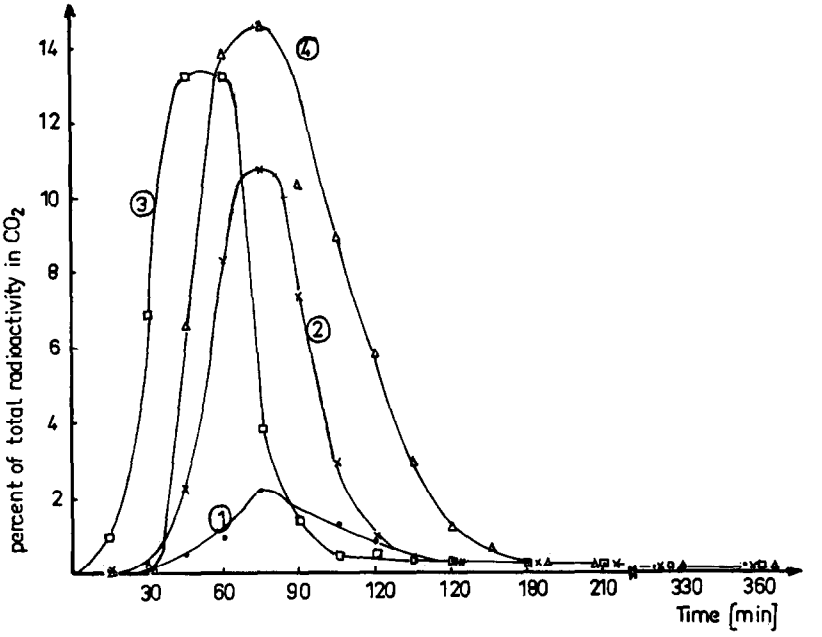

Fig. 1. Kinetics of $^{14}CO_2$ formation from carboxyl-labelled *p*-hydroxybenzoic acid (1), vanillic acid (2) and syringic acid (4) by cell suspension cultures of mungbean. (3) presents $^{14}CO_2$ formation by culture (2) after addition of a second batch of vanillic acid. Total $^{14}CO_2$ production: 1 9.4%, 2 34.8%, 3 42.5%, 4 65.5%.

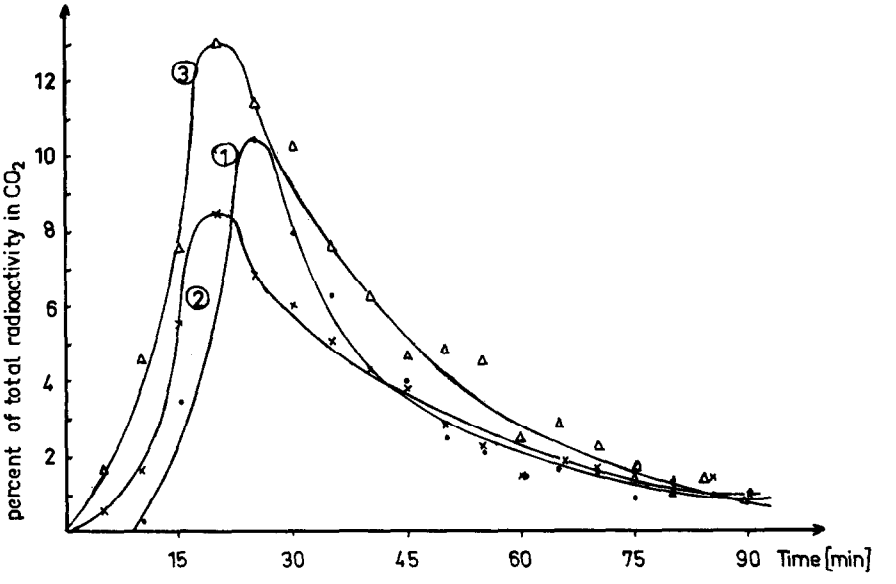

Fig. 2. Kinetics of $^{14}CO_2$ formation from carboxyl-labelled vanillic acid (1), syringic acid ((2) first addition of substrate), and syringic acid ((3) second addition of substrate) by cell suspension cultures of soybean. Total $^{14}CO_2$ production: (1) 57.1%, (2) 57%, (3) 88%.

3. Results and discussion

When catechol-(U-^{14}C) and specifically ^{14}C-labelled benzoic and cinnamic acids are inoculated into cell suspension cultures of mungbean and soybean a strong formation of $^{14}CO_2$ was observed. Tables 1 and 2 summarize the results and it is obvious that both cultures posses the capability for ring fission especially demonstrated by the values for catechol, protocatechuic acid and caffeic acid. The actual degree of ring fission can be assumed to be much higher than indicated by the $^{14}CO_2$ values because the metabolites formed are likely to be preferentially incorporated into cellular constituents. The rate of decarboxylation is very pronounced in case of the multiply substituted benzoic acids. This phenomenon parallels earlier observations with intact plant material [7, 8, 10] though the values obtained with cell cultures are significantly higher. The observed demethylation reactions further provide evidence that plants can split alkyl-aryl-ether bonds also in benzoic acids [9, 20]. In general, the soybean cell cultures when compared with the mungbean cultures are more effective in metabolizing the various substrates used. The only exception being benzoic acid itself which is not oxidized by the soybean cell suspensions.

The very high degree of decarboxylation observed with substituted benzoic acids (tables 1 and 2) has led to kinetic experiments. Fig. 1 shows the rate of $^{14}CO_2$ formation from carboxyl-labelled *p*-hydroxybenzoic acid, vanillic acid and syringic acid by mungbean cell suspensions. After approximately 20 min $^{14}CO_2$ production begins and maximum decarboxylation is reached at 75 min. Some sort of induction process is indicated by the observation that a culture which has once received vanillic acid (curve 2) can after a 24 hr lapse of time oxidize vanillic acid practically immediately and to a greater extent (curve 4). Soybean cell suspensions very efficiently metabolize carboxyl-labelled vanillic and syringic acid as shown by curves 1 and 2 in fig. 2. Within 90 min some 57% of the radioactivity has been evolved as $^{14}CO_2$ with maximum velocity between 20 and 25 min. A second batch of syringic acid applied to the same culture after 24 hr is again oxidized much more rapidly (curve 3). Differences in metabolism of benzoic acids by soybean cell suspensions are obvious by the results obtained with *p*-hydroxybenzoic acid and protocatechuic acid as shown in fig. 3. In the case of protocatechuic acid maximum $^{14}CO_2$ formation is not reached before 40 min while *p*-hydroxybenzoic acid is oxidized at a constant rate (compare table 2) not influenced by repeated addi-

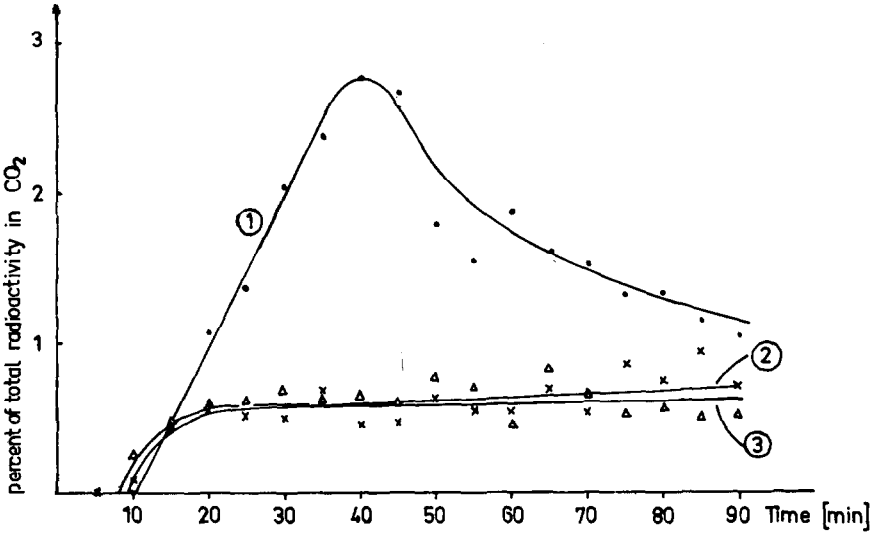

Fig. 3. Kinetics of $^{14}CO_2$ formation from carboxyl-labelled protocatechuic acid ① and *p*-hydroxybenzoic acid after first ② and second ③ addition of substrate by cell suspension cultures of soybean. Total $^{14}CO_2$ production: ① 25.9%, ② 10.0%, ③ 9.9%.

tion of new substrate (curves 2 and 3). In the latter case other rate-controlling steps prior to decarboxylation may be expected.

In summary, our results show that plant cell cultures are useful systems for studying catabolic reactions of secondary plant products. Further studies on the elucidation of the pathways concerned as well as induction and purification of the enzymes are in progress.

Acknowledgements

We thank Dr. K. Hahlbrock and Mr. T. Lindl for providing the soybean cell cultures. This work was supported by Deutsche Forschungsgemeinschaft (SFB 46).

References

[1] M.A. Zaprometov, Proc. Acad. Sci. USSR 125 (1959) 1359.
[2] M.H. Zenk, Ber. Deutsch. Botan. Ges. 80 (1967) 573.
[3] H. Grisebach and W. Barz, Naturwissenschaften 56 (1969) 538.
[4] R. Kickuth, Naturwissenschaften 57 (1970) 55.
[5] K.O. Vollmer, H.J. Reisener and H. Grisebach, Biochem. Biophys. Res. Commun. 21 (1965) 221.
[6] K.H. Bolkart and M.H. Zenk, Z. Pflanzenphysiol. 59 (1968) 439.
[7] H. Harms, H. Söchtig and K. Haider, Plant Soil 31 (1969) 129.
[8] H. Harms, H. Söchtig and K. Haider, Plant Soil 31 (1969) 257.
[9] W. Barz and H. Grisebach, Z. Naturforsch. 22b (1967) 627.
[10] H. Harms, H. Söchtig and K. Haider, Z. Pflanzenphysiol. 64 (1971) 437.
[11] S.Z. El-Basyouni, D. Chen, R.K. Ibrahim. A.C. Neish and G.H.N. Towers, Phytochem. 3 (1964) 485.
[12] W. Barz, Ch. Adamek and J. Berlin, Phytochem. 9 (1970) 1745.
[13] R. Janistyn, W. Barz and R. Pohl, Z. Naturforsch. (1971), in press.
[14] J. Berlin adn W. Barz, Planta (Berlin) (1971), in press.
[15] B.E. Ellis and G.H.N. Towers, Phytochem. 9 (1970) 1457.
[16] K. Hahlbrock, E. Kuhlen and T. Lindl, Planta (Berlin) (1971), in press.
[17] H. Jeffay and J. Alvarez, Analyt. Chem. 33 (1961) 612.
[18] K. Haider and S.U. Lim, J. Labelled Compds. 1 (1965) 294.
[19] K. Haider, J. Labelled Compds. 2 (1966) 174.
[20] J. Ebel, H. Achenbach, W. Barz and H. Grisebach, Biochim. Biophys. Acta 215 (1970) 203.