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IN VIVO CHARACTERIZATION OF CATECHOL RING-CLEAVAGE IN CELL CULTURES OF *GLYCINE MAX*

S. PRASAD and B. E. ELLIS

Guelph-Waterloo Centre for Graduate Work in Chemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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Abstract—No evidence could be obtained for the involvement of dioxygenases in the generation of $^{14}CO_2$ from catechol-[^{14}C] observed in soybean cell cultures. Culture filtrates incubated with catechol and H_2O_2 were, however, able to catalyze the ring-cleavage, as was horseradish peroxidase.

INTRODUCTION

Aromatic ring-cleavage reactions form a recently-established part of the metabolism of the products of the shikimic acid pathway in plants [1]. The existence of these reactions has, in some cases, been deduced from the results of biosynthetic studies using specifically labelled precursors [2–5], while in others release of $^{14}CO_2$ from ring-labelled aromatic substrates has been monitored [6, 7]. Enzyme extracts catalyzing intradiol ring-cleavage of 2,3-dihydroxybenzoic acid and extradiol ring-cleavage of DOPA have been prepared from *Tecoma stans* [8] and *Stizolobium hassjoo* [9], respectively. Both systems appear to involve a dioxygenase-type cleavage similar to the well-characterized analogous systems in micro-organisms.

The most widely tested substrate for ring-cleavage in plants has been catechol, which has been shown to undergo a limited catabolism to CO_2 in all plant cell cultures examined [7]. This compound is accumulated in a number of plant genera (*Gaultheria* [10], *Allium* [11], *Salix* [12], *Populus* [13]) and has also been suggested as an intermediate in the catabolism of phenol in plants [14]. In the latter studies, label from phenol-[^{14}C] fed to intact plants could later be detected in muconic acid, among the other compounds. This result implied the operation in these plants of an intradiol dioxygenase system similar to microbial pyrocatechase. When recent attempts in this laboratory to detect pyrocatechase and metapyrocatechase activity in enzyme extracts of several plant cell cultures proved unsuccessful, a more detailed study was made of the release of $^{14}CO_2$ from such cultures during incubation with catechol-[U-^{14}C].

RESULTS AND DISCUSSION

Most of the present work was carried out using a fast growing cell line of soybean hypocotyl tissue (*Glycine max* cv Amsoy) which had been maintained as a suspension culture for over 12 months.

In order to establish the optimum conditions for catechol ring-cleavage in the soybean cultures, two parameters were initially examined; the culture age and the concentration of administered catechol. Cultures were sampled at various times after inoculation and 1.5 g fr. wt of tissue was removed aseptically with 20 ml of the cell-free medium. This suspension was shaken with 5 μM catechol-[^{14}C] in a sealed flask for 3 hr and the radioactivity in the respired CO_2 then measured. The catabolic acitivity showed no notable maximum or minimum but remained relatively constant throughout the growth cycle, unlike many enzyme activities involved in aromatic metabolism in plants [15]. Even cultures in advanced static phase (14–16 days after inoculation) which have undergone an appreciable loss of dry wt and are in irreversible metabolic decline retained an active catechol ring-cleavage capacity.

When the concentration of catechol in the medium was increased in the range of 10 μM to 500 μM, several effects were observed (Table 1). At lower concentrations, the $^{14}CO_2$ release was concentrated in the initial 8 hr incubation period but with increasing concentrations the release was both reduced and delayed.

Autoradiograms prepared of the ethanol-soluble extractives from each culture after 2-D TLC showed that virtually the only labelled compound extracted from the 500 μM culture was catechol glucoside (R_f values and reaction with diazotized *p*-nitroaniline in comparison with authentic material). As the administered catechol concentration was lowered, however, the prominence of the glucoside declined and 4 metabolites of lower mobility became more prominent. At 10 μM catechol, the glucoside was a very minor component in a complex mixture of labelled compounds dominated by the 4 new metabolites. The latter all gave colour reactions with diazotized *p*-nitroaniline typical of phenolic derivatives. In no case was radioactivity detectable at the position of *cis*, *cis*-muconic acid (or its isomers), γ-oxalocrotonic acid or free catechol on the chromatograms (results not shown).

The correlation between the appearance of the new metabolites and the rapid $^{14}CO_2$ release might suggest a functional relationship between these phenomena. The *Glycine* metabolites, however, appear to retain an intact phenolic character and, in similar experiments, suspension cultures of *Nicotiana*, *Phaseolus* and *Lycopersicon* were found to convert 10 μM catechol-[^{14}C] to both $^{14}CO_2$ and a chromatographically distinctive set of labelled

Table 1. Effects of increasing catechol concentration and of prior exposure to catechol on catechol ring-cleavage in soybean suspension cultures

Concentration of catechol-[U-^{14}C] (10^6 dpm)	dpm ^{14}C recovered in CO_2 0–8 hr	8–16 hr	16–20 hr	total	%^{14}C remaining in medium after 20 hr
10 μM	11 300	8030	1340	20 700	9
50 μM	8560	7940	1090	17 600	7
150 μM	1120	1910	2650	5680	11
500 μM	260	500	120	880	69
5 μM	16 300†				6†
5 μM pretreated*	13 700†				6†

Values represent the average of duplicate experiments.

* Pretreated cultures were administered non-radioactive catechol in increasing amounts (total of 9 μmol) over a period of 9 days prior to the addition of radioactive catechol.

† Incubation for 4 hr.

derivatives. It therefore seems unlikely that the metabolites represent the products of dioxygenase-catalyzed ring-cleavage.

Induction of enhanced catabolism of aromatic substrates is a common phenomenon in microbial systems [16]. Soybean cell cultures have also been reported to respond to administration of hydroxybenzoic acids by decarboxylating or demethylating the compound more rapidly in subsequent incubations than in the initial test [6]. When soybean cultures in the present study were exposed to a series of additions of 5 μM catechol-[^{14}C] there was no indication of a similar short term induction and prior exposure of the cultures to a total of 9 μmol non-radioactive catechol over a period of 9 days also produced no enhancement of the catechol ring-cleavage (Table 1).

Soybean cell cultures have previously been shown to catabolize not only catechol but also caffeic acid [6], 3,4-dihydroxybenzoic acid [6] and DOPA [17]. The latter 3 structures appear much more frequently in plant metabolsim than catechol and it might be expected that any plant catabolic system would preferentially catalyze ring-cleavage of the phenylpropanoid or benzoic acid structures. Higher rates of $^{14}CO_2$ production from these compounds than from catechol have in fact, been observed in cell cultures [6, 17]. A series of competition experiments were carried out in which soybean cultures were incubated with 5 μM catechol-[^{14}C] and a 20-fold excess of another potential ring-cleavage substrate. The production of $^{14}CO_2$ was monitored after 2 hr and 4 hr but since the results at both time intervals yielded the same pattern, only the total $^{14}CO_2$ recovery over 4hr is reported (Table 2).

Table 2. Effect of excess substituted catechols on ring-cleavage of catechol-[U-^{14}C] in soybean cultures

Added substrate (0.1 mM)	dpm $^{14}CO_2$ recovered from catechol-[U-^{14}C] (10^6dpm; 5 μM) during a 4 hr incubation
	22 200 ± 5200*
Caffeic acid	28 800
2,3-Dihydroxybenzoic acid	15 100
3,4-Dihydroxybenzoic acid	17 600
3,4-Dihydroxybenzoic acid	17 400
DOPA	16 400
Chlorogenic acid	21 000
p-Nitrocatechol	22 800
Catechol	4 100

* Control value represents mean of 8 experiments; other values are average duplicates.

Rather than indicating any preference for the more common dihydroxy aromatic substrates, the results show a notable specificity for catechol as substrate in this system. While two potential substrates depressed $^{14}CO_2$ release slightly below the range of control values, none was as effective as catechol itself at the same concentration.

After consistently failing to detect label in chromatographically re-isolated *cis,cis*-muconic acid or γ-oxalocrotonic acid added before or after short-term (10–30 min) or long-term (3–24 hr) incubations of catechol-[^{14}C] with soybean cultures (results not shown), a series of trapping experiments were undertaken in which catechol-[^{14}C] ring-cleavage was monitored in the presence and absence of a 100-fold molar excess of either intradiol ring-cleavage intermediates (*cis-cis*-muconic acid plus β-ketoadipic acid) or extradiol intermediates (γ-oxalocrotonic acid plus 2-keto-4-methylbutyrolactone) during a 4 hr incubation. This same format was used to test cell cultures of *Nicotiana tabacum*, *Lycopersicon esculentum* and *Phaseolus vulgaris* (Table 3).

Rather than reducing the release of $^{14}CO_2$ from catechol-[^{14}C], the presence of the ring-cleavage intermediates frequently stimulated the process in each species tested. Although the rate of uptake of the dicarboxylic acids was not determined, *cic,cis*-muconic acid was easily detected (UV absorbance, R_f values) in the cell extracts when these were chromatographed by 2D TLC. Autoradiography, however, revealed no detectable label in the muconic acid, nor in the expected location of the other trapping intermediates, and the autoradiographic patterns were essentially identical in the control and experimental incubations.

Table 3. Effect of excess intradiol and extradiol ring-cleavage intermediates on catechol-[U-^{14}C] ring cleavage in plant cell cultures.

Species	% ^{14}C recovered in CO_2 during a 4 hr incubation with catechol-[U-^{14}C] (5 μM) Compounds added		
	None	*cis,cis*-muconic acid (0.5 mM) + β-ketoadipic acid (0.5 mM)	γ-oxalocrotonic acid (0.5 mM) + 2-keto-4-methylbutyrolactone (0.5 mM)
Glycine max	1.1	1.6	2.8
Phaseolus vulgaris	0.8	0.9	2.5
Nicotiana tabacum	1.9	1.9	1.3
Lycopersicon esculentum	0.6	0.9	0.6

The results again suggest that the administered trapping compounds either are not intermediates in the reactions responsible for the observed ring-cleavage or are not reaching the relevant metabolite pools.

The response of the soybean catechol ring-cleavage system to a number of inhibitors was examined during a 2 hr incubation. KCN inhibited $^{14}CO_2$ release 98% at a 1 mM concentration but NaN_3 at the same concentration was consistently stimulatory (145% of controls). Ethylxanthate and phenylthiourea are known catechol oxidase inhibitors [18, 19] and 0.1 mM concentrations inhibited the $^{14}CO_2$ release 64% and 38% respectively. All known aromatic dioxygenase mechanisms require O_2 but the results of flushing the cultures with an inert gas before (20 min) and during the incubation period were surprisingly variable despite care to remove O_2 from the flushing gas. The relative $^{14}CO_2$ release values ranged from 7 to 49% of controls run at the same time in a number of experiments using both N_2 and Ar.

This apparent lack of an absolute dependence on O_2 suggested that the latter might be utilized only in some indirect fashion for ring-cleavage. When a N_2-flushed culture was incubated with catechol-[^{14}C] plus H_2O_2, the $^{14}CO_2$ release was stimulated to levels equal to and greater than those previously observed in an air atmosphere (Table 4). Peroxidase has recently been shown to be responsible for the previously observed rapid decarboxylation of hydroxybenzoic acids in soybean cell cultures [21], and it was also noted that the extracellular peroxidase in the cultures was potentially a major site of the decarboxylating activity. A similar pattern was observed in the present work, with H_2O_2-supplemented cell-free culture medium carrying out a rapid ring-cleavage of catechol (Table 5). Cells transferred to fresh medium were also capable of catalyzing the ring-cleavage, although at a reduced rate, possibly through the action of cell-wall bound or intracellular peroxidase. Peroxidase was readily detected in filtered culture medium (0.06–1.2 μM U ml) but very little H_2O_2 could be measured (<1 μg/ml) in freshly filtered samples. Recent reports of H_2O_2 [21] and peroxide-generating enzyme systems in plant cell walls [22, 23] suggest that a major site of peroxidase/H_2O_2 catalyzed reactions may be at the surface of the cell.

Not surprisingly, horseradish peroxidase also proved to be an excellent catalyst for catechol ring-fission when provided with H_2O_2 at pH 7 (Table 5). Unlike the hydroxybenzoic acid decarboxylation reported [20], however, the ring-fission reaction requires H_2O_2 in amounts much beyond the stoichiometric level before maximum CO_2 release is attained. The detailed properties of this peroxidase-catalyzed aromatic ring-cleavage are under investigation.

These studies do not support the original hypothesis that dioxygenases are responsible for the $^{14}CO_2$ release observed during catechol-[^{14}C] incubations with plant cell cultures. No direct ring-cleavage products predictable from an intradiol or extradiol cleavage mode could be detected in the cells during incubation with catechol and a large excess of these products failed to trap label from catechol-[^{14}C], or to reduce $^{14}CO_2$ release, during a period of active $^{14}CO_2$ evolution. The extracellular (and presumably cell-wall and intracellular) peroxidases of *Glycine max* cultures are, however, apparently capable of catalyzing catechol ring-cleavage by

Table 4. Effect of H_2O_2 on anaerobic catechol ring-cleavage in soybean cell cultures

Incubation conditions	dpm $^{14}CO_2$ recovered from catechol-[U-^{14}C] (10^6dpm; 5 μM) incubated with the cell suspension for 1 hr
N_2 atmosphere	530
+ H_2O_2 (0.05 mM)	1700
+ H_2O_2 (0.5 mM)	2920
+ H_2O_2 (5 mM)	7380
+ H_2O_2 (50 mM)	12420
+ H_2O_2 (150 mM)	19050
+ H_2O_2 (300 mM)	27650

Table 5. Catechol ring-cleavage by H_2O_2-supplemented cell-free culture medium and horseradish peroxidase

Incubation		dpm $^{14}CO_2$ recovered from catechol-[U-^{14}C] (50 μM; 5 × 10^5 dpm) after 2 hr
Soybean cells in original medium		3970
Washed cells in fresh medium		1970
Cell-free original medium		270
	+ 50 mM H_2O_2	11500
Horseradish peroxidase*		60
	+ 0.01 mM H_2O_2	670
	+ 1.0 mM H_2O_2	8700
	+ 100 mM H_2O_2	20900
	+ 350 mM H_2O_2	37700
	+ 450 mM H_2O_2	37000

* Horseradish peroxidase (3 μM U) was incubated in 0.1 mM Na phosphate pH 7.

some as yet undefined mechanism if sufficient H_2O_2 is present. It seems likely that the same process is functioning in the other cell cultures examined but the possible relevance of this reaction to intracellular aromatic ring catabolism remains to be be determined.

EXPERIMENTAL

Chemicals. Catechol-[U-^{14}C] was prepared enzymatically from phenol-[U-^{14}C] as described previously [24]. β-Ketoadipic acid, *cis,cis*-muconic acid, γ-oxalocrotonic acid and 2-keto-4-methylbutyrolactone were generously provided by Dr. W. C Evans, Department of Biochemistry and Soil Science, University College of North Wales. Horseradish peroxidase (3500 μM U/mg) was obtained from Sigma. All other compounds were reagent grade commercial products.

Cell suspension cultures. Aseptically-grown hypocotyl sections were used to establish cultures of *Glycine max* cv Amsoy, *Nicotiana tabacum* L. cv Xanthi, *Lycopersicon esculentum* Mill cv Chico III and *Phaseolus vulgaris* cv Topcrop on a modified B5 medium [25]. Pretreatment of soybean cultures was carried out by adding 1 μmol of catechol aseptically to a 50 ml culture 3, 5 and 7 days after inoculation, followed by 2 μmol on day 8 and 4 μmol on day 9.

Monitoring catechol ring-cleavage. Aliquots (20 ml) of suspension culture were transferred aseptically to 125 ml conical flasks fitted for flushing the suspension with a sterile air stream The serum stoppered side arms allowed subsequent addition of filter sterilized solns to the suspension. The incubations were carried out in the dark on a gyrotary shaker (120 rpm) and the suspensions flushed with air (30 ml/min) at appropriate intervals. The respiratory CO_2 was trapped in phenylethylamine and counted. Cultures to be incubated in an inert atmosphere were flushed with N_2 or Ar which had been scrubbed in a potassium pyrogallate tower. Cells were 'washed' when necessary by filtering them aseptically and resuspending in 3 changes of fr. B5 medium The original medium was passed through a 0 22 μm Millipore filter and designated 'cell-free culture medium.'

Cell extracts. The incubated cultures were filtered, rinsed and extracted at once with boiling 80% EtOH The conc extract was chromatographed by 2D TLC on Avicel (PhOH–H_2O–HOAc, 80 · 20 : 1 and *n*-BuOH–propionic acid–H_2O, 15 : 7 : 10) with and without authentic carriers (*cis,cis*-muconic acid, γ-oxalocrotonic acid, catechol). In some cases, aliquots were extracted 3× with Et_2O before and after acidification and the conc extracts separated by Si gel TLC (hexane–Et_2O–HOAc, 5 : 20 : 1) and cellulose TLE (0.05 M NH_4OAc pH 3.5). All chromatograms and electrophoretograms were laid on GAF HR-1000 X-ray film for 1–4 weeks before visual evaluation and comparison with the original plates. Separated compounds were detected by their *A* at 254 nm in UV light and reaction with diazotized *p*-nitroaniline, 2,4-dinitrophenylhydrazine, I_2 vapour or bromcresol green.

Assays. Peroxidase and H_2O_2 in filtered growth medium were assayed by standard procedures [26].

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