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TIME-COURSE TRACER STUDIES ON THE METABOLISM OF CINNAMIC ACID IN *CESTRUM POEPPIGII*

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Key Word Index - *Cestrum poeppigii*; Solanaceae; cinnamic acid; HPLC; polyphenols; chlorogenic acid; 1-cinnamyl-β-D-glucose; glucose esters.

Abstract—Time-course tracer studies were performed on the metabolism of *trans*-cinnamic acid-[3-^{14}C] and *trans*-*p*-coumaric acid-[2-^{14}C] in the leaves of *Cestrum poeppigii*, resulting in a dynamic picture of the turnover of several plant polyphenols. Using HPLC for the analysis of these products, it is shown that 1-cinnamyl-,1-*p*-coumaryl- and 1-caffeyl-β-D-glucose are of prime importance in phenylpropane metabolism. They have high biosynthetic and turnover rates and they are readily converted to other cinnamyl derivatives when fed in labeled form.

INTRODUCTION

In earlier studies on the metabolism of cinnamic, *p*-coumaric and caffeic acid and their quinic acid esters [1, 2], we found that most of these compounds are metabolically active. The biosynthesis of the commonly occurring glucose esters of cinnamic acids was not yet investigated in our laboratory. Our attention has now focused on 1-cinnamyl-β-D-glucose, 1-*p*-coumaryl-β-D-glucose and 1-caffeyl-β-D-glucose. In the past, unlabeled and labeled precursors have been fed to plant material to study the biosynthesis of these esters [3–6]. Extensive experiments with cinnamic acid-[2-^{14}C] have been performed by Kojima and Uritani [7–9]. They concluded that 1-cinnamyl-β-D-glucose is actively turned over in the plant material used, and that it is the first intermediate in the biosynthesis of chlorogenic acid. In *in vitro* experiments on the biosynthesis of glucose esters, Corner and Swain [10] and Macheix [11] obtained an enzyme preparation that converts cinnamic acids to their 1-glucose esters in the presence of UDPG. We have now applied time-course tracer studies with cinnamic acid-[3-^{14}C] to leaves of *Cestrum poeppigii* plants to obtain a better insight in the metabolic activity and fate of these derivatives.

RESULTS AND DISCUSSION

Chromatographic analysis

Wulf and Nagel [12] have found that reverse phase columns do not separate complex polyphenol mixtures, although good separations can be obtained within a given group. To analyse our plant extracts, a clean up procedure was needed, followed by a group separation. An anion exchange column of the cellulose type was used to separate the complex mixtures into an acidic and a non-acidic fraction.

Time-course tracer studies with trans-*cinnamic acid-*[3-^{14}C]

After feeding a short pulse of *trans*-cinnamic acid-[3-^{14}C] to our plant material, the radioactivity is distributed over the extractable and non-extractable portion of the plant. Figure 1 shows how this distribution varies with time. In 10 hr, a rather stable ratio is reached: about 25% of the cinnamic acid-[3-^{14}C] fed to the plant material has been converted to insoluble products and 75% is found in the soluble polyphenol pool. The nature of these insoluble compounds has not been determined yet. It is doubtful that they are precursors of soluble plant polyphenols, in view of their slow labeling characteristics in comparison with the latter: the soluble products under investigation in this work are labeled in the first hours of infiltration (cf. Fig. 1). They do not accumulate radioactivity, as the amount rapidly decreases towards the end of the experiment. The distribution pattern of radioactivity in the soluble pool is represented schematically: rapidly labeled substances are shown in Fig. 2, the more slowly labeled in Fig. 3. Cinnamylglucose and *p*-coumaric acid are the first soluble products formed, and they have very high turnover rates [cf. 2].

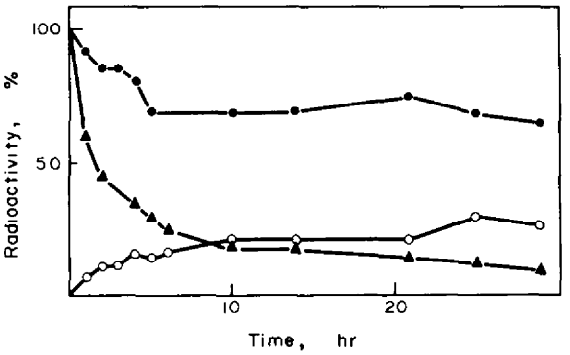

Fig. 1. Distribution of radioactivity in the soluble (●) and insoluble part (○) of *Cestrum poeppigii* leaves after pulse labeling with *t*-cinnamic acid-[3-^{14}C]. The amount of radioactivity is expressed in %, 100% being the total amount of radioactivity administered. The third graph (▲) represents the total amount of radioactivity found in cinnamic + *p*-coumaric + chlorogenic acid + cinnamylglucose + *p*-coumarylglucose + caffeylglucose.

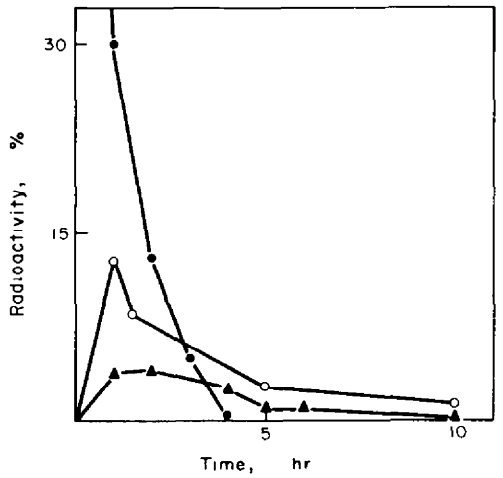

Fig. 2. Rapidly labeled compounds after administration of *t*-cinnamic acid-[3-^{14}C] to *Cestrum poeppigii* leaves. Cinnamic acid (●), *p*-coumaric acid (▲) and 1-cinnamyl-β-D-glucose (○). (Radioactivity is expressed in %, total amount of radioactivity in the ethanolic extract is 100%.)

The *p*-coumarylglucose pool contains most of the radioactivity, reaching a maximum *ca* 4 hr after the pulse (Fig. 3). 1-Caffeyl-β-D-glucose and chlorogenic acid are labeled more slowly, reaching maximum values after 10 and 14 hr, respectively. Towards the end of the experiment, label accumulates mainly in one strongly polar component of unknown structure. Quantities of products measured in *Cestrum poeppigii* leaves in this experiment, in μg/g fresh plant material, are: chlorogenic acid, 300–600; caffeylglucose, 200–500; *p*-coumarylglucose ~20. Cinnamylglucose, *p*-coumaric and caffeic acid could not be detected in UV. Specific activities are typically in the order: *p*-coumarylglucose > caffeylglucose (*ca* 30 times), caffeylglucose > chlorogenic acid (*ca* 10 times).

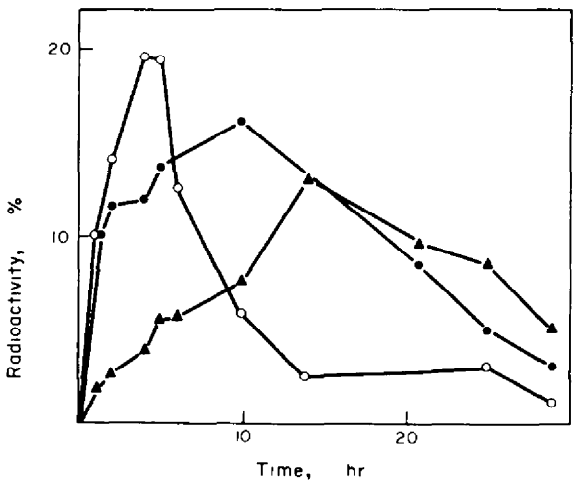

Fig. 3. Kinetics of labeling of 1-*p*-coumaryl-β-D-glucose (○), 1-caffeyl-β-D-glucose (●) and chlorogenic acid (▲) after pulse labeling with *t*-cinnamic acid-[3-^{14}C]. (Radioactivity is expressed in %, total amount of radioactivity in the ethanolic extract is 100%.)

* The numbering of the quinic acid ring has been adapted to the new nomenclature rules. In previous publications, this compound was named 3-*O*-*p*-coumarylquinic acid.

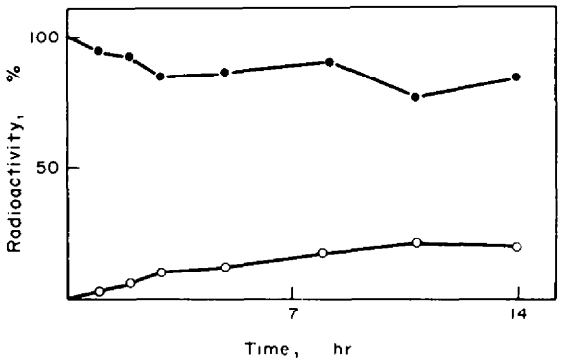

Fig. 4. Distribution of radioactivity (100% is the total amount of radioactivity fed) in the soluble (●) and insoluble (○) fractions of *Cestrum poeppigii* leaves after pulse labeling with *t*-*p*-coumaric acid-[2-^{14}C].

Time-course tracer studies with trans-p-*coumaric acid*-[2-^{14}C]

p-Coumaric acid-[2-^{14}C] is incorporated into insoluble pools with the same rate and to the same extent as cinnamic acid-[3-^{14}C] (Fig. 4). *p*-Coumarylglucose is the first metabolite formed in the soluble fraction (Fig. 5). It is immediately converted to other polyphenols. Radioactive caffeic acid could not be detected in this experiment.

Chlorogenic acid is the main metabolite formed at longer infiltration times, together with 5-*O*-*p*-coumarylquinic acid* and caffeylglucose. Specific activities of all components in order of magnitude are: *p*-coumaric acid

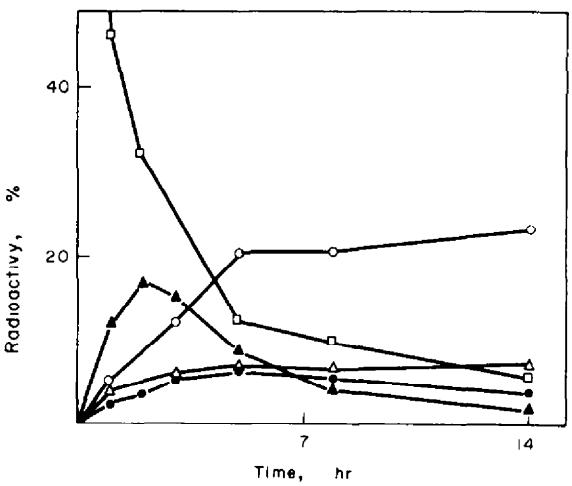

Fig. 5. Distribution of radioactivity in *Cestrum poeppigii* leaves after pulse labeling with *t*-*p*-coumaric acid-[2-^{14}C]. Radioactivity in *p*-coumaric acid (□), 1-*p*-coumaryl-β-D-glucose (▲), 1-caffeyl-β-D-glucose (△), 5-*O*-*p*-coumarylquinic acid (●) and chlorogenic acid (○), is expressed in % relative to the total amount administered being 100%.

> *p*-coumarylglucose > *p*-coumarylquinic acid > caffeylglucose > chlorogenic acid. From Fig. 5, one might conclude that the investigated products have no active turnover: only *p*-coumarylglucose shows fast biosynthesis and breakdown rates after feeding labeled *p*-coumaric acid. In this case, the slow conversion of labeled *p*-coumaric acid has to be taken into account: in the analogous experiment with cinnamic acid-[3-^{14}C], the latter precursor is consumed about twice as fast by the plant leaves.

Other feeding experiments

Five hours after feeding a short pulse of 9 μCi 1-(*p*-coumaryl-[3-^{14}C])-β-D-glucose to *Cestrum poeppigii* leaves, 75% of the label was found in soluble products and 25% in insoluble material. Label was converted to chlorogenic acid (9.4%), *p*-coumaric acid (4.3%) and 5-*O*-*p*-coumarylquinic acid (3.7%). Two other unknown products are labeled (16 and 5.7%). 5.3 μCi 1-(caffeyl-[3-^{14}C])-β-D-glucose was fed as a short pulse and the leaves were analysed five hours later. Only a few compounds were labeled; the highest amount of radioactivity was found in chlorogenic acid (14.5%).

Both glucose esters are efficiently converted to other polyphenols and they are better precursors for chlorogenic acid than *p*-coumaric and caffeic acid. They are comparable with cinnamic and 5-*O*-*p*-coumarylquinic acid in this respect, for which we obtained conversion efficiencies to chlorogenic acid of *ca* 10 and 16%, respectively [2]. In combination with our previous work on the biosynthesis of chlorogenic acid [2], these results point to the pathway: cinnamic acid → *p*-coumaric acid → *p*-coumarylglucose → 5-*O*-*p*-coumarylquinic acid → chlorogenic acid, for the biosynthesis of chlorogenic acid.

We can conclude that esterification with glucose is an important reaction for the free cinnamic acids fed to our plant material. The glucose esters formed are further metabolized. When fed in a labeled form, they are quickly and efficiently converted to chlorogenic acid and other polyphenols.

EXPERIMENTAL

Plant material. Cestrum poeppigii plants, all derived from one clone were grown outdoors and the mature plants analysed in summer.

Feeding techniques. Full grown leaves were placed with their ends in the infiltration solns, radioactive tracer in H_2O. The labeled product was taken up in about 20 min and H_2O was given for a further period of time. At certain time intervals, 4 leaves were extracted and analysed.

Radioactive precursors. Cinnamic acid-[3-^{14}C] was obtained from IRE. Specific activity: 50 μCi/μM. *p*-Coumaric acid-[2-^{14}C] was synthesized using *p*-hydroxybenzaldehyde and malonic acid-[2-^{14}C] 1-(*p*-coumaryl-[3-^{14}C])-β- -glucose [13] was obtained from *Cestrum poeppigii* leaves after a 5 hr infiltration with cinnamic acid-[3-^{14}C]. It was extracted and purified chromatographically on a preparative scale with a reversed phase column. 1-(Caffeyl-[3-^{14}C])-β- -glucose was obtained in the same way.

Reference products. 1-*p*-Coumaryl-β-D-glucose and 1- caffeyl-β-D-glucose were synthesized as described by Birkofer *et al.* [14, method b]. The reaction mixture thus obtained for caffeylglucose was purified by preparative HPLC on a reversed phase column, and the structure of the pure products was verified by NMR. 1-Cinnamyl-β-D-glucose could be synthesized in mg quantities as follows: 4 g ethyl-1-thio-β-D-glucopyranoside [15] and 24 g cinnamic acid silver salt were refluxed in 170 ml acetonitrile for 30 hr. Ag^+ was removed with H_2S, and the resulting reaction mixture purified by preparative HPLC.

Isolation procedures. An 80% EtOH extract was concd *in vacuo* to *ca* 3 ml and centrifuged at 25000 *g* for 10 min. The supernatant contained the phenolics of interest. The residues of concn and centrifugation were dissolved in CH_2Cl_2–H_2O. This mixture was concd *in vacuo* to *ca* 3 ml and the resulting H_2O phase centrifuged at 25000 *g* for 10 min. The supernatant was added to that first obtained and both were concd to 3 ml. H_2O was added to a final vol. of 5 ml. This extract was injected on a small preparative DEAE column (9 mm i.d. × 5 cm length), which had been equilibrated with 0.01 M acetate buffer (pH = 6.0). The column qas eluted with this buffer soln at a constant flow of 0.5 ml/min. This fraction contained the glucose esters. Then, 50 ml 0.01 N H_2SO_4 was passed over the column at a flow of 1 ml/min. This fraction contained the phenolic acids. A new column was packed for each analysis. Both fractions were concd *in vacuo* to a small vol. (*ca* 1 ml) and H_2O was added to obtain a vol. of exactly 2 ml. This can be injected into a HPLC analytical column.

HPLC analysis. We used a 4.6 mm ID column of 25 cm length, packed with Lichrosorb RP8 (10 μm, Merck) reversed phase material (slurry pack method).

This column was placed in a HP 1080A apparatus from Hewlett Packard and eluted with a solvent gradient: 11% B isochratically during 5 min and a linear gradient to 63% B in 25 min at a flow rate of 4 ml/min. A is 0.001 N H_2SO_4 and B is MeOH.

50 μl quantities were injected. The flow was monitored with a UV detector at 280 nm and also with a radiometric detector (Berthold). The flow cell of the latter was packed with cerium-activated lithium glass (cell vol.: 200 μl). If radioactivity were to be measured quantitatively, eluting products would be collected, mixed with a liquid scintillation counting mixture, and counted in a liquid scintillation counter.

Identification of glucose esters. For the identification of the 3 glucose esters, the radioactively labelled products obtained from feeding exts with cinnamic acid-[3-^{14}C] were purified with HPLC. The chromatographically pure products were easily hydrolysed with β-glucosidase, and the labeled aglucone identified by its retention behaviour on a reversed phase column. The retention vols of the glucose esters on HPLC were identical with synthetic products. 1-Caffeyl-β-D-glucose is found in relatively high quantities in *Cestrum poeppigii* leaves. 300 μg of this ester were isolated using reversed phase column chromatography.

The ester was completely hydrolysed in 0.1 M H_2SO_4 at 100°. The amount of caffeic acid was determined with HPLC (UV detection). Using glucose oxidase (Merck) for the determination of the sugar residue, a *ca* 1:1 ratio was found.

Radioactivity measurements of insoluble material. The insoluble residues were washed thoroughly with EtOH, and dried in an oven at 60° to constant wt, which was typically 10% of the fr. wt. The dry residue was burned in a Packard tri-carb sample oxidizer and counted in a liquid scintillation counter.

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