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KINETIC STUDY OF POSSIBLE INTERMEDIATES IN THE BIOSYNTHESIS OF CHLOROGENIC ACID IN *CESTRUM POEPPIGII*

L. NAGELS and F. PARMENTIER
Laboratorium voor Algemene Scheikunde, Rijksuniversitair Centrum, Antwerpen, België

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Key Word Index—*Cestrum poeppigii*; Solanaceae; biosynthesis; chlorogenic acid; phenolic acids; HPLC of phenolic acids.

Abstract—*p*-Coumaric and 3-*O*-*p*-coumarylquinic acid seem to be important precursors of chlorogenic acid in the leaves of *Cestrum poeppigii*. 3-*O*-Cinnamylquinic acid, which has a very small metabolic activity, is of little importance in this respect. The kinetics of incorporation of radioactivity from *t*-cinnamic acid-3-[^{14}C] into *p*-coumaric, 3-*O*-*p*-coumarylquinic, chlorogenic and 3-*O*-cinnamylquinic acid showed that the biosynthetic rates for these products decrease in the order shown. For *p*-coumaric acid, which has a markedly high metabolic activity, a turnover rate of 28 μg/hr and per gram fresh plant leaf, was calculated. Some trapping experiments with caffeic acid, and the acids mentioned above and using either *t*-cinnamic acid-3-[^{14}C] or *p*-coumaric acid-2-[^{14}C] as precursor, are discussed. A HPLC method for the rapid determination of phenolic acids in plant extracts, is described.

INTRODUCTION

In a previous publication [1] we described some dynamic aspects of caffeic acid formation in *Cestrum poeppigii*. Kinetic studies have been carried out for chlorogenic acid and isochlorogenic acids [2], and for certain insoluble hydroxy cinnamic acid derivatives [3,4]. The latter products could be important precursors of lignins and/or soluble derivatives [5], such as chlorogenic acid. Kojima *et al.* [6] also described the kinetics of formation and breakdown of β-1-cinnamyl-D-glucose [7], to support the idea that this product may be an important precursor of chlorogenic acid.

Indeed, although conversions from cinnamic, *p*-coumaric and caffeic acids to chlorogenic acid have been observed *in vivo*, it is not certain that the free acids themselves are important intermediates. The efficiency with which they are converted is in the order: cinnamic > *p*-coumaric > caffeic acid [8,9], the reverse of that which would be expected. Such data should be considered with care, however, because they may be strongly dependent on infiltration time, as will become evident later in this article. Other shortcomings of the *in vivo* tracer method are possible differences between the endogenous and exogenous (or supplied) pools of free acids.

Time-course tracer studies on the level of free cinnamic acids have not yet been carried out. These acids do not occur naturally in detectable amounts in *C. poeppigii*, but nevertheless we started kinetic studies, using trapping techniques. Indeed, a small pool size does not imply small turn-over rates, as will be shown for *p*-coumaric and *p*-coumarylquinic acids.

The quinic acid esters of cinnamic and *p*-coumaric acid have also been claimed to be chlorogenic acid precursors by Levy and Zucker [10]. Trapping experiments with 3-*O*-cinnamylquinic acid were performed by Hanson [11], using potato tuber slices, and showed that the hypothesis is not valid for the cinnamic acid ester, unless it is assumed that exogenously supplied acid does not reach the active metabolic pool. Hanson found that the 3-*O*-*p*-coumarylquinic acid fraction contained more radioactivity, and had a higher specific activity than chlorogenic acid. Conclusions about a possible precursor–product relationship on these data only, are premature, as long as the comparison of specific activities is not extended to time-course tracer studies.

RESULTS

Chromatographic analysis

Column chromatography of plant phenolic acids has hitherto been a time-consuming means for the quantitative determination of phenols in plant extracts, even though a system with a good resolution and a wide range of applicability has been described [12]. We have used the same phase system and support material as described [12], in a HPLC system, which gave a high plate number, a much shorter analysis time and ease of manipulation. The characteristics of the column, expressed as the number of theoretical plates for chlorogenic acid, and the resolution between two peaks, are completely comparable to those of the classical (low pressure) column [12]. Under the conditions described (see Experimental) we obtained an analysis in 60 min. *p*-Coumaric, caffeic, chlorogenic, 3-*O*-cinnamylquinic and 3-*O*-*p*-coumarylquinic acid had elution volumes of 33, 49, 83, 49 and 65 ml respectively with 1.55 ml/min., and a pressure of 200 psi. Elution of the compounds is quantitative, and a linear correlation between peak area and quantity (over 1–20 μg) was found, using a 20 μl flow-cell in an UV detector at 280 nm. The column has been used about 100 times with the same filling, without change in efficiency. Reproducibility, expressed as the % standard deviation on the elution volume of chlorogenic acid is 3,5%. One difference from the column of Hanson and

Table 1. Trapping experiments

No.°	Weight of leaves (g)	Inf. time (hr)	Tracer†		Trapping compound†				Chlorogenic acid		
				μCi fed		Rad. act. (% of uptake)	μM	$\frac{\mu Ci}{\mu M} \times 10^3$	Rad. act. (% of uptake)	μM	$\frac{\mu Ci}{\mu M} \times 10^3$
A1	3.1	2.2	C	5.0	—	—	—	—	8.2	4.4	93
A2	2.5	2.2	C	5.0	CQA	1.5	2.1	35	10	5.0	100
B1	2.2	1.5	C	5.0	—	—	—	—	7.8	6.5	60
B2	2.5	1.5	C	5.0	pCQA	5.0	2.1	120	11	7.5	76
B3	1.6	2.0	pC	4.4	—	—	—	—	4.5	4.5	44
B4	2.1	2.0	pC	4.4	pCQA	2.7	1.9	64	3.6	6.9	24
C		1.0	pC	3.5	CA	2.1	0.40	100	2.7	6.2	15
D		1.5	pC	3.5	CA	1.5	1.3	40	5.7	7.7	25
§E1	1.6	7.0	C	3.5	CA	5.0	0.78	220	3.4	1.2	95
§E2	1.3	7.0	C	10	CA	2.4	0.59	400	2.3	1.3	180

§E1 and E2 are two results that were taken out of our 1974 publication, for comparison. †CQA = 3-*O*-cinnamylquinic acid, pCQA = 3-*O*-*p*-coumarylquinic acid, CA = caffeic acid. C = cinnamic acid 50 μCi/μM; pC = *p*-coumaric acid 0.84 μCi/μM. °Infiltrations A → D were performed within 14 days (Aug–Sept. 1974) to avoid changes in metabolism due to seasonal variation. Experiments marked with the same letter were done on the same day, and using the same plant material.

Zucker [12] is that *trans* and *cis* isomers can be separated. The R_{cg} factor [12], can be extended to the HPLC column.

Trapping experiments

Trapping experiments give us information about the conversion efficiency of the precursor to the trapping substance (trapper) and also about the relation between the latter and the ultimate product, chlorogenic acid. As we artificially enlarge the pool of the trapping product in the plant, it will accumulate radioactivity from the fed precursor (cinnamic or *p*-coumaric acid). If the product is an important intermediate in the biosynthesis of chlorogenic acid, enlarging its pool should retard radioactivity on its way to chlorogenic acid. This effect is greatest in the interval immediately following the fed pulse of radioactivity (in the first hour or so of infiltration). If the product is not an important intermediate, there will be no such effect on the labeling of chlorogenic acid: radioactivity will reach this acid as fast, and with the same efficiency, as in a reference experiment (normal pool size of the studied product). The results of experiments to study such effects, and to measure conversion efficiencies from precursor to trapper are shown in Table 1.

Several comments arise from these results. Consider first the experiments with cinnamic acid-3-[^{14}C] as precursor.

(a) Using either 3-*O*-cinnamylquinic acid or 3-*O*-*p*-coumarylquinic acid as a trapper has no effect on the conversion efficiency from cinnamic to chlorogenic acid. Indeed, the radioactivity accumulating in chlorogenic acid is even slightly higher when the trapping acids were present.

(b) 3-*O*-Cinnamylquinic acid always had a lower specific activity than chlorogenic acid (cf. also kinetic experiments), whereas the reverse holds for 3-*O*-*p*-coumarylquinic acid.

(c) Finally, it can be seen that parahydroxylation of cinnamic acid proceeds much faster than esterification with quinic acid (A_2 in Table 1 and kinetic experiment with *p*-coumaric acid).

Experiments with *p*-coumaric acid-2-[^{14}C] as precursor showed similar trends.

(a) Exogenously supplied cinnamic acid is a better precursor than *p*-coumaric acid for chlorogenic acid, *p*-coumarylquinic acid and caffeic acid.

(b) Using *p*-coumarylquinic or caffeic acid as trappers has no striking effect on the conversion efficiency from *p*-coumaric acid-2-[^{14}C] to chlorogenic acid, as compared to the reference infiltration.

(c) The specific activities of *p*-coumarylquinic and caffeic acid were higher than that of chlorogenic acid.

Kinetic experiments

The only radioactive tracer used for kinetic experiments was cinnamic acid-3-[^{14}C]. All kinetics were measured in the same way as for the trapping experiments which means that all the measured products had an artificially enlarged pool size at all times during the kinetic experiment, except chlorogenic acid, because this is the only product occurring in detectable amounts in *C. poeppigii*.

In these experiments, we tried to obtain a picture of the dynamic behaviour of the products under investigation, in order to study their interconversions.

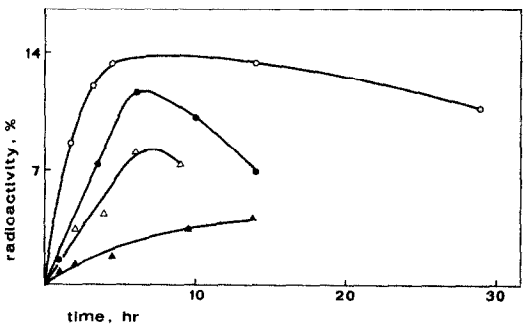

Fig. 1. Kinetics of chlorogenic acid formation with cinnamic acid-3-[^{14}C] as tracer. The four graphs represent the labeling of chlorogenic acid without trapping products (○), or with *p*-coumaric (▲), *p*-coumarylquinic (●), and cinnamylquinic acid (△) as a trapping substance. The kinetics of these trapping products, measured at the same time, are shown in figures 2 and 3. The amounts of chlorogenic acid found at different times were as follows: ○: 2.84, 3.64, 4.2, 3.2, 2.82 mg; ●: 1.14, 1.15, 1.6, 1.91 mg; △: 0.74, 1.24, 0.71, 0.74 mg; and ▲: 2.61, 3.52, 1.54, 2.95, 2.69 mg. The expt without trapping substances (○) was performed 17 September 1974, using 4 leaves and 4.68 μCi cinnamic acid per analysis.

The results for the labeling of chlorogenic acid and of *p*-coumaric acid are shown in Fig. 1 and 2 respectively. As can be seen, without trapping agents the labeling of chlorogenic acid reaches a maximum after about 6 hours. After this period, there is little change; chlorogenic acid in *C. poeppigii* seems to have a small turn-over. (Fig. 1). The same cannot be said about *p*-coumaric acid, which appears to have much higher rates of biosynthesis and breakdown (Fig. 2). The turn-over rate is about 28 μg/h/g fresh weight which means a half life of approximately 4.3 hours for *p*-coumaric acid under the conditions given.

After 2 hr the *p*-coumaric acid pool contains 44% of the administered radioactivity. The total flux of radioactivity through the *p*-coumaric pool is given by:

$$\text{flux} = \text{turn-over} \times \int_{t=o}^{t=\infty} \text{spec. act.} \times dt.$$

(assuming that the turn-over is constant over the observed period). The value of this integral was determined from the specific activity-time curve and gives a value for the flux of 2.23 μCi, or 50% of the administered radioactivity, which may be a good estimation of the amount of cinnamic acid that is converted to *p*-coumaric acid. In this experiment with *p*-coumaric acid, the chlorogenic acid fraction is labelled much more slowly than the same fraction in the absence of high *p*-coumaric acid concentrations (see Fig. 1). Also, the quantity of radioactivity reaching the chlorogenic acid pool, is significantly lower when there is a high *p*-coumaric acid pool.

The kinetics of 3-*O*-cinnamylquinic acid formation, is completely different from that of *p*-coumaric acid (Fig. 4). This product is slowly labelled, and no turnover is visible. Conversion is low, the cinnamic ester reaching a maximum of 3.5% at the end of the experiment. The results are in agreement with the other trapping experiments with cinnamylquinic acid, except that in this kinetic experiment, chlorogenic acid is labelled to a somewhat lesser extent (Fig. 1). 3-*O*-*p*-Coumarylquinic acid shows a fast biosynthetic rate (intermediate between the rates for *p*-coumaric and chlorogenic acid), the highest amount of radioactivity being reached in about 5 hr (Fig. 3). We calculated a turnover rate of 77 μg/h/g fr. plant material. The calculated flux of radioactivity through this pool was about 17% of the total amount administered. The measurements for chlorogenic acid indicate that an extension of the *p*-coumarylquinic acid pool with exogenous supplied material has no significant effect on the labeling of chlorogenic acid.

After feeding labelled *p*-coumarylquinic acid (0.25 μCi, 0.75 μCi/μM), to plant leaves (1.60 g fresh weight) during 2.17 hr., chlorogenic acid was found to contain 16% of the administered radioactivity.

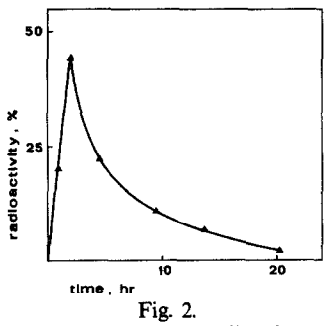

Fig. 2.

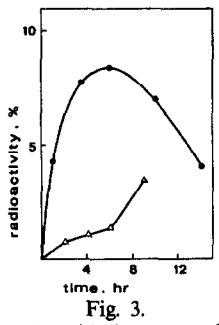

Fig. 3.

Fig. 2. Kinetics of labeling for *p*-coumaric acid. Amounts of *p*-coumaric acid are as follows: 0.75, 0.71, 0.52, 0.49, 0.83, 0.66 mg. The expt was performed on 9 Sept. 1974, using 4 leaves and 4,44 μCi of cinnamic acid per analysis.

Fig. 3. Kinetics of labeling for 3-*O*-*p*-coumarylquinic (●) and 3-*O*-cinnamylquinic acid (△). Amounts of product: (●): 0.65, 0.48, 0.55, 0.71, 0.93 mg; rad. act. fed: 11,4 μCi (△): 0.52, 0.78, 0.59, 0.37 mg; rad. act. fed: 14,3 μCi Experiment dates: (○) 24 September 1974, (△) 1 October 1974. 2 Leaves were used per analysis.

DISCUSSION

Different hypothesis for the biosynthesis of chlorogenic acid have been formulated, but most authors agree that labeling of chlorogenic acid from cinnamic acid proceeds rapidly, in fact within a few hours. It is evident therefore, that the precursors must be labelled still more quickly. We have found that the labeling of cinnamylquinic and caffeic acid [1] is slower than the labeling of chlorogenic acid.

Furthermore, the specific activities of these acids are lower than the specific activity of chlorogenic acid in the kinetic experiments. Thus, cinnamylquinic acid and caffeic acid can play only a minor role in chlorogenic acid biosynthesis. *p*-Coumaric and *p*-coumarylquinic acid, on the other hand, possess the required kinetic qualities of a precursor of chlorogenic acid, if their overall radioactivity–time kinetics is compared with those of chlorogenic acid formation. Under normal conditions (low concentration of *p*-coumaric and *p*-coumarylquinic acid), these products must be labelled faster still, as we retard the radioactivity by using trapping conditions. So, our measurements of the time required to obtain maximum labeling for these two acids (2 and 5 hr respectively), must be considered to be much longer than those occurring in the normal system without external feeding.

The measured turn-over rate for *p*-coumaric acid will also be higher in our system than it is in normal conditions. The effect of high *p*-coumaric acid concentrations on the kinetics of chlorogenic acid labeling is just that expected if the compound is a good precursor. Another factor in favour of the hypothesis that *p*-coumaric and 3-*O*-*p*-coumarylquinic acid are precursors of chlorogenic acid, is that their specific activities are higher than that for chlorogenic acid. However, two experimental facts seem to disagree with this hypothesis. First, we obtained only poor conversions from *p*-coumaric acid 2-[^{14}C] to 3-*O*-*p*-coumarylquinic and to chlorogenic acid. No simple explanation can be found for this, and further work on this subject will be necessary. Secondly, raising the *p*-coumarylquinic acid concentration has no significant effect on the conversion efficiencies from cinnamic to chlorogenic acid, in contrast to the experiments with *p*-coumaric acid. If chlorogenic acid was quantitatively formed by hydroxylation of 3-*O*-*p*-coumarylquinic acid, we would expect a significant retardation of the radioactivity on its way to the chlorogenic acid pool, when using 3-*O*-*p*-coumarylquinic acid as a trapper. On the other hand, if the acid is only partly converted to chlorogenic acid, the effect of reducing the radioactivity can be very small. We know that about 17% of the radioactivity from cinnamate passes through this *p*-coumaric acid ester pool, and that only 16% of the ester is converted to chlorogenic acid. This means that the amount of radioactivity (from cinnamic acid), that is converted to chloro-

genic acid via the cinnamic →→ 3-*O*-*p*-coumarylquinic → chlorogenic acid pathway, is only about 3% of the fed radioactivity. So, at a given moment, the pool of 3-*O*-*p*-coumarylquinic acid can hold a maximum of about 3% of the fed radioactivity from the chlorogenic acid pool. Such effects are too small to be distinguished. The fact that we measure only small effects, supports the idea that 3-*O*-*p*-coumarylquinic acid is only partly converted to chlorogenic acid. A good estimate of the contribution of the cinnamic →→ 3-*O*-*p*-coumarylquinic → chlorogenic acid pathway from our measurements, is that about one third to one fourth of the label in chlorogenic acid is derived from this pathway.

EXPERIMENTAL

Plant material. Mature *Cestrum poeppigii* plants, which were all derived from one clone, were grown outdoors, and analysed in summer.

Feeding techniques. In all expts, full grown leaves (2–4 leaves per experiment) were placed with their severed ends in the infiltration solns (undiluted radioactive tracer in H_2O). The solns were taken up by the plant leaves in a short time (about 15 min.). In reference infiltrations (without trapping substances, H_2O was given for a further period of time (1–2 hr). When a trapping substance (*p*-coumaric, caffeic, 3-*O*-cinnamylquinic or 3-*O*-*p*-coumarylquinic acid), was used, it was given as a saturated solution in H_2O to the cut leaf ends for about 2 hr before the pulse of radioactive precursor. This was followed by the solution of the trapping compound for varying periods of time, depending on the kind of experiment (1 to 2 hours for a normal, and variable time intervals for a kinetic experiment).

Radioactive precursors. We used cinnamic acid-3-[^{14}C], obtained from CEA. Specific activity: 50 μCi/μM. *p*-Coumaric acid-2-[^{14}C] was synthesized using *p*-hydroxybenzaldehyde and malonic acid-2-[^{14}C], as described by Vorsatz [13]. The reaction mixture was purified by extraction and chromatography: the partition column described by Hanson and Zucker [12] was used for this purpose. Radiochemical purity of the isolated *trans*-*p*-coumaric acid-2-[^{14}C] was verified with the HPLC column, the eluent being continuously monitored by both a UV and a radioactivity detector. Only the trans-isomer was observed as the reaction product. 3-*O*-Cinnamylquinic and 3-*O*-*p*-coumarylquinic acid were synthesized as described [14,15]. The end products were purified by partition column chromatography, and their structure confirmed by NMR, UV, IR, MS, MP and R_{cg} factors [12].

Isolation of compounds from plant extracts. 80% EtOH was used to extract leaves (20–50 ml/g) under reflux. The filtrate was mixed with 0.5 g Si gel, and the mixture evaporated to dryness. The gel was placed on top of a preparative column. Any residue remaining in the container was dissolved in EtOH, and adsorbed onto another 0.5 g Si gel, dried, and transferred to the column. 2.0 ml 0.5 N H_2SO_4 were used to wet this dry layer of Si gel. (Column 1.5 × 13 cm; with 12 g Si gel (Macherey-Nagel, 50–200 μm) and 6 ml 0.5 N H_2SO_4 as stationary phase). Elution was started with 60 ml cyclohexane–$CHCl_3$ (1:3; equilibrated with 0.5 N H_2SO_4). This fraction is discarded. A second fraction, which is eluted with 250 ml tert-BuOH–$CHCl_3$ (2:3; v/v, equilibrated with 0.5 N H_2SO_4) containing all phenols of interest, was concentrated ($t < 40°$) to *ca* 0.5 ml. H_2O was added to a final vol of exactly 2.0 ml. After centrifugation (10000 rpm for 10 min.), 30 μl of the supernatant were injected onto the HPLC column. The HPLC column consisted of a glass column, 2.7 × 270 mm, filled with Si gel (Merckosorb SI 60, 40 μm), using a dry tamping technique. 0.5 N H_2SO_4 was used as a stationary phase and was added to the column after packing. The amount needed was variable to some degree, without influencing the results. The following gradient was used throughout to separate the phenols we are interested in: solvent A, cyclohexane–$CHCl_3$ (1:9; v/v, equilibrated with 0.5 N H_2SO_4); solvent B, tert-BuOH–$CHCl_3$ (3:7; v/v, equilibrated with 0.5 N H_2SO_4). 0–15 ml: 100% A, 15–93 ml: linear gradient to 70% B. Pressure was 200 psi. During the gradient run, mixing of solvents A and B causes some H_2O condensation, and this enlarges the vol of the stationary phase on the support material, which may eventually cause trouble, when using a UV detector. Most of this water was held up in the mixing chambers used between pump and solvent reservoirs. The solvent are pumped at a speed of 1.55 ml/min.

Quantitative determinations. The amount of each phenol was determined by area measurements of the UV signal; radioactivity was measured by collecting total peak fractions and subsequent counting in a liquid scintillation counter.

Quantitative and purity tests. Several 3-*O*-*p*-coumarylquinic and 3-*O*-cinnamylquinic acid fractions, labelled in trapping experiments, were purified first with a low pressure partition column [13]. The pure products were hydrolysed in 1 N HCl at 100° for 1 hr. A sample of the reaction mixture was immediately injected on the HPLC. The elution of *p*-coumaric and cinnamic acid respectively, was continuously followed with UV and radioactivity monitors. Coincidence of the two signals was checked to control the radiochemical purity. This was done for all investigated products.

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