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CHLOROGENIC ACID BIOSYNTHESIS IN *CESTRUM POEPPIGII*

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Abstract—The chlorogenic acid content of *Cestrum poeppigii*, and its ability to form the acid from labelled *t*-cinnamic acid, was determined at different stages of growth In contrast to mature plants, young plants showed great seasonal variation in their chlorogenic acid content The incorporation of radioactivity from *t*-cinnamic into chlorogenic acid also differed greatly during the growth period Trapping experiments with caffeic and p-coumaric acids were performed to study the effect of large pools of these acids on the incorporation of *t*-cinnamic acid-3-[^{14}C] into chlorogenic acid The kinetics of incorporation exclude a major role for caffeic acid in the biosynthesis of chlorogenic acid

INTRODUCTION

LITTLE is known about the variation in the concentration and the rate of biosynthesis of chlorogenic acid during growth of plants. Some studies have been carried out on the effect of photoperiodic induction,[1,2] but no change was found in the turnover of chlorogenic acid after induction of flowering. However, the concentration and turn-over of the acid were found to be dependent on the part of the plant that was analyzed.[3,4]

The biosynthetic pathway from cinnamic to chlorogenic acid is also not yet fully understood. Several products have been shown to be precursors of chlorogenic acid but their relative importance is not clear,[5,6] although, caffeic acid was generally a relatively bad precursor, whereas p-coumaric acid was comparable to, or even more efficient than *t*-cinnamic acid.

Trapping experiments have provided additional information [6] For example, Steck found a good conversion of *t*-cinnamic into p-coumaric acid, but very little into caffeic acid. Some authors have also investigated the kinetics of chlorogenic acid formation in plants using labelled *t*-cinnamic acid as precursor. A simple first order kinetics was found by Taylor *et al.*,[7] in *Xanthium* leaf disks. Colonna *et al.*,[8] on the other hand, found an increase of radioactivity during the first few hours of infiltration, followed by a constant level during the following 50 hr, in coffee plants. Caffeic acid derivatives, both soluble and insoluble,

[1] ZUCKER, M, NITSCH, C and NITSCH, J P (1965) *Am J Botany* **52,** 271
[2] TAYLOR, A O and ZUCKER, M (1968) *Z Pflanzenphysiol Bd* **59,** 103
[3] TAYLOR, A O (1968) *Phytochemistry* **7,** 63
[4] KOEPPE, D E, ROHRBAUGH, L M, RICE, E L and WENDER, S H (1970) *Phytochemistry* **9,** 297
[5] RUNECKLES, V C (1963) *Can J Biochem Physiol* **41,** 2249
[6] STECK, W (1968) *Phytochemistry* **7,** 1711
[7] TAYLOR, A O, ZUCKER, M (1966) *Plant Physiol* **41,** 1350
[8] COLONNA, J P, BOUDET, A and GAUTHERET, A (1971) *C R Acad Sc Paris* **272,** 952

have been studied by several authors in kinetic experiments, because of their possible role as lignin precursors. Nothing, however, is known about the kinetics of free caffeic acid formation. Whether this acid is an important precursor of chlorogenic acid or not, has not yet been established.

We tried to get a better insight into all these problems and especially the caffeic acid–chlorogenic acid relation, by observations on *cestrum poeppigii*. Seasonal variations, trapping experiments and kinetic studies have all been carried out.

TABLE 1. AMOUNT OF CHLOROGENIC ACID AND INCORPORATION OF LABEL FROM *t*-CINNAMIC ACID-3-[^{14}C] IN YOUNG AND MATURE PLANTS OF *Cestrum poeppigii*

Date of experiment (1973)	Weight of shoots (g)	Uptake of *t*-cinnamic acid-(μCi)	Chlorogenic acid formed (μg)	(μg/g)	$\frac{\mu Ci}{\mu M}$	Radioact (% of uptake)
Young plants						
18 Jan	1·00	8·30	0	0		0
25 May	1·34	22·8	0	0		0
1 Aug	1·50	10·1	757	505	0·274	5·85
16 Aug	1·05	5·05	625	595	0·0322	1·13
24 Sept	0·796	4·00	172	216	0·00183	0·318
Mature plants						
26 June	1·16	10·12	273	235	0·313	1·34
1 Aug	1·67	10·06	436	261	0·439	5·42
16 Aug	1·46	5·05	372	255	0·0325	0·675
24 Sept	1·39	4·04	342	246	0·0376	0·906

RESULTS

Seasonal variation in chlorogenic acid biosynthesis

The chlorogenic acid content and the incorporation of radioactivity from labelled cinnamic acid were determined quantitatively in sections of shoots from young and old plants. As can be seen in Table 1, young plants show great differences in the concentration of chlorogenic acid during development. The results confirm earlier data, that the metabolic activity of the plant is at its maximum at the beginning of August.[9] Such variations are not encountered in mature plants where the chlorogenic acid concentration is practically constant. The percentages of radioactivity, incorporated from cinnamic into chlorogenic acid, however, are variable in both young and mature plants and there is no correlation with concentration. Thus, while the percentage of incorporation differs by a factor of eight in the infiltrations performed on Aug. 1, 1973 and on Aug. 16, 1973 (mature plant series), only 2·5% variation of concentration was noted (Table 1). This indicates that differences in the rates of biosynthesis do not automatically infer great changes in concentration. The same holds, to a somewhat lesser extent, for the younger plants.

Trapping experiments

The results of the trapping experiments are given in Table 2. The most striking fact is, that we found no detectable amounts of chlorogenic acid in the trapping experiments with p-coumaric acid, although analyses of the same mature plant showed normal quantities

[9] PARMENTIER, F. (1964) Thesis, University Ghent.

of the ester. This result may reflect a regulating effect of p-coumaric acid on the biosynthesis or turnover of chlorogenic acid. If the biosynthesis of chlorogenic acid is blocked by high p-coumaric acid concentrations, and the chlorogenic acid disappears almost completely after 9 hr (total experiment time), we may conclude that chlorogenic acid must have a high turnover. The amount of radioactivity found in p-coumaric acid is high (13 and 6%) in agreement with the experiences of Steck.[6]

TABLE 2 THE EFFECT OF THE ADDITION OF UNLABELLED P-COUMARIC ACID AND CAFFEIC ACID ON THE INCORPORATION OF *t*-CINNAMIC ACID-3-[^{14}C] INTO CHLOROGENIC ACID

Weight of shoots (g)	Uptake (μCi)	Trapping compound				Chlorogenic acid		
			(μg)	$\frac{\mu Ci}{\mu M}$	Rad act (% of uptake	(μg)	$\frac{\mu Ci}{\mu M}$	Rad. act (% of uptake
First series								
1 60	3 31	p-coumaric	256	0 277	13 1	0	—	0
1 57	3 46	caffeic	141	0 221	5 03	437	0 0952	3 41
1 51	3 40	—	—	—	—	397	0 216	7·11
Second series								
1 10	8 92	p-coumaric	172	0 511	6 00	0	—	0
1 34	10 1	caffeic	106	0 405	2 36	449	0 18	2 27

Steck found little radioactivity in chlorogenic acid (0·031%) in an analogous trapping experiment which he attributed to p-coumaric acid taking up most of the radioactivity and not being further hydroxylated. As reported earlier,[10] we also found a good conversion from *t*-cinnamic to o-coumaric acid In contrast to Steck,[6] we found a good conversion of *t*-cinnamic into caffeic acid. When we used caffeic acid as a trapping compound, less radioactivity was found in chlorogenic acid, relative to the reference infiltration (3·41% vs 7·11%). At first sight, this might mean that caffeic acid lies on a direct pathway between cinnamic and chlorogenic acid but this hypothesis was not confirmed by the further kinetic experiments. The effect is, therefore, not yet clear.

TABLE 3 VARIATION IN THE AMOUNT AND SPECIFIC ACTIVITY OF CHLOROGENIC AND CAFFEIC ACID DURING THE COURSE OF THE KINETIC EXPERIMENT

Infiltration Time (hr)	Chlorogenic acid		Caffeic acid	
	(μg)	(μCi/μM) × 10^2	(μg)	(μCi/μM) × 10^2
4	385	6 94	155	4 75
8	415	5 22	550	2 43
11	410	4 46	650	3 76
17	395	4 18	408	5 00
26	425	4 43	435	3 15

Kinetic experiments

Our further experiments on the kinetics of caffeic acid biosynthesis (Table 3), show that caffeic acid cannot be an intermediate on a major pathway from cinnamic to chlorogenic acid. Its specific activity is lower than that of chlorogenic acid, except after 17 hr, and comparison of the kinetics of the formation of both products excludes a simple precursor-product relation

[10] NAGELS L and PARMENTIER, F (1973) *Arch Int Physiol Biochim* **81**, 733

Chlorogenic acid gets labelled more quickly to start with, and then part of the radioactivity is lost with a constant amount of radioactivity being reached about 10 hr after the end of the pulse The caffeic acid pool on the contrary contains its maximum amount of radioactivity 10 hr after the cinnamic acid pulse. This means that, although radioactivity reaches the caffeic acid pool quite quickly, it is still being actively synthesized 8–11 hr after the short pulse of labelled cinnamic acid The results do not however rule out the possibility that caffeic acid is a secondary precursor of chlorogenic acid

EXPERIMENTAL

Plant Material Mature plants of *Cestrum poeppigii* height about 190 cm were grown in the greenhouse They had all been derived from one clone One was taken as the clone for the young plants, set in Nov 1972 and also grown in the greenhouse At the end of the survey (Sept 1973), these young plants had a height of about 50 cm

Feeding techniques Plant shoots with 2 leaves, were cut at about 20 cm from the top of a branch (Weight 1·1 6 g) They were placed in a soln containing 1 2 ml H_2O about 1 mg radioactive cinnamic acid, and an equimolar amount of D(−) quinic acid After this solution had been absorbed, H_2O was supplied to the shoots These were illuminated with 2800 lx, by Osram L-Fluora lamps The total infiltration time was 7 hr For the trapping experiments saturated solutions of the 'trapping' acids were fed in the light to plant shoots for 2 hr Then, a short pulse of *t*-cinnamic acid-3-^{14}C (50 μCi μM) in 250 μl H_2O was given After this, the shoot was replaced in light in the solns of the "trapping" acid for 7 hr more The same conditions were employed in the reference infiltration with H_2O instead of the acids Shoots were taken from mature plants only In the kinetic experiment 20 fully-grown leaves derived from one branch of a mature plant were used The cut leaf ends were placed in the infiltration solutions They were treated in exactly the same way as described above for the trapping experiments with caffeic acid (e g infiltration of a saturated soln of caffeic acid for 2 hr a 15 min pulse of *t*-cinnamic acid-3-^{14}C, and replacement in the saturated acid solution for variable times) four leaves were withdrawn randomly and analyzed each time

Isolation and purification techniques In all experiments, the plant material to be examined was boiled in 80% EtOH for 15 min , mixed, boiled for another 15 min , and insoluble material filtered-off Purification of the extract was performed in two steps using preparative and analytical column chromatography The preparative column used was the same as described earlier [10] The analytical column used was analogous to the column described by Hanson and Zucker [11]

Qualitative analysis. Elution from the analytical column was followed continuously with a spectrophotometer, equipped with a flow cell For some extracts the column eluent was split, one part passing through an UV spectrophotometer (at 320 nm), the other part mixed with scintillator soln and passed through a scintillation spectrometer flow cell All fractions which were of interest were evaporated to dryness and dissolved in EtOH The amount of compound was assayed spectrophotometrically Radioactivity was measured in a liquid scintillation counter

Identification and purity tests The R_{cg} value of the eluting compounds [11] was very useful as a means of qualitative identification In practically all chromatograms, chlorogenic acid and the fed p-coumaric and caffeic acids were the only phenols eluting from the analytical column U V spectra in EtOH and EtOH + NaOH were taken for each fraction sampled and the fluorescence noted All fractions were also subjected to TLC on cellulose plates (20 × 5 cm) with BuOH–HoAc-H_2O (4 1 5), as eluent Radiochemical purity was verified by passing the chromatograms through a radiochromatogramscanner The amount and radioactivity were finally determined when the samples were found to be pure enough for further investigation

[11] HANSON, K R and ZUCKER M (1963) *J Biol Chem* **238**, 1105