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METABOLISM OF CINNAMIC ACID IN PLANTS: CHLOROGENIC ACID FORMATION*

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Abstract—Rapid, efficient synthesis of chlorogenic acid from cinnamate was observed in leaves of plants of several genera. Use of labelled cinnamate, *p*-coumarate and caffeate showed two pathways were operative in this synthesis in tobacco. Trapping experiments indicated the major route to be: cinnamic acid → *p*-coumaric acid → *p*-coumaroylquinic acid → chlorogenic acid, and the secondary route cinnamic acid → *p*-coumaric acid → caffeic acid → chlorogenic acid. The metabolism of chlorogenic acid is discussed.

INTRODUCTION

CHLOROGENIC acid (3-*O*-caffeoyl-D-quinic acid; II in Fig. 1) occurs widely among the higher plants, usually along with isomers such as neochlorogenic acid (5-*O*-caffeoyl-D-quinic acid) and analogous coumaroyl and feruloyl compounds.[1] This class of compounds has been collectively termed "chlorogenates", but the term "chlorogenoids" seems more apt, and will be used below. "Chlorogenoids" might well include not only quinic acid derivatives but also those of shikimic and other closely allied acids. The term "chlorogenate" ought to be used only in the customary chemical sense to designate salts and esters of the chlorogenic (caffeoylquinic) acids.

The biosynthesis of chlorogenoids has been studied previously.[2–5] Phenylalanine and cinnamic acid are recognized precursors, but some aspects of the pathways beyond cinnamate are still unclear. Using potato tuber disks, Hanson and Zucker[2] obtained a cell-free system able to convert 3-*O*-*p*-coumaroylquinic acid (Fig. 1, I) to chlorogenic acid. Their suggestion that 3-*O*-cinnamoylquinic acid might also be an intermediate was not supported by the later work of Hanson,[3] who indicated nonetheless that *p*-coumaroylquinic acid was a precursor. The possibility of direct combination of caffeic and quinic acids was considered by Runeckles,[4] but in his tobacco leaf disks caffeic acid was poorly incorporated into chlorogenic acid. Recently, Gamborg[5] obtained good transformation of caffeic acid in potato cell cultures, so that caffeate has to be re-evaluated as a possible precursor of chlorogenoids. This report describes some experiments carried out in this connexion.

RESULTS

Tobacco leaf disks were fed [2-^{14}C]cinnamic acid, [2-^{14}C]*p*-coumaric acid, [2-^{14}C]caffeic acid and 4-glucosido-[2-^{14}C]caffeic acid to determine their effectiveness as precursors of

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[1] E. SONDHEIMER, *Botan. Rev.* **30**, 667 (1964).
[2] K. R. HANSON and M. ZUCKER, *J. Biol. Chem.* **238**, 1105 (1963).
[3] K. R. HANSON, *Phytochem.* **5**, 491 (1966).
[4] V. C. RUNECKLES, *Can. J. Biochem. Physiol.* **41**, 2249 (1963).
[5] O. L. GAMBORG, *Can. J. Biochem.* **45**, 1451 (1967).

chlorogenic acid. The results are shown in Tables 1 and 2. In each case hydrolysis with pectinase showed essentially all the radioactivity of the isolated chlorogenic acid to be located in the caffeoyl part of the molecule. Parallel feedings of whole detached leaves gave very similar results. In every instance the amount of label incorporated into neochlorogenic acid was slight, although this compound was present in tobacco leaves in one-quarter the concentration of chlorogenic acid.

Labelled chlorogenic acid was obtained from all of these substrates, so two trapping experiments were then carried out to clarify the roles of *p*-coumaric and caffeic acids. In the first experiment, a nearly saturated solution of *p*-coumaric acid was fed for 20 min to a detached tobacco leaf, then [2-^{14}C]cinnamic acid was added to the solution and the mixture fed 1 hr; finally the cut leaf end was replaced in *p*-coumaric acid solution for a further 2 hr. The specific activities of some metabolites were measured. In the second experiment caffeic acid was used in place of *p*-coumaric. Table 3 summarizes the results of these experiments.

TABLE 1. UPTAKE AND UTILIZATION OF LABELLED SUBSTRATES BY TOBACCO LEAF DISKS AFTER 4 hr OF METABOLISM. THE FINAL COLUMN REPRESENTS WATER-SOLUBLES LESS RESIDUAL SUBSTRATE THEREIN. TWO EXPERIMENTS WITH CINNAMIC ACID ARE REPORTED AS (*a*) AND (*b*)

Compound fed	μc/mmole	Offered (μc)	Uptake (μc)	Water-solubles (μc)	Metabolites (μc)
[2-^{14}C] Cinnamic acid					
(*a*)	2000	1·68	0·18	0·120	0·088
(*b*)	1050	1·68	0·16	0·081	0·039
[2-^{14}C]*p*-coumaric acid	943	2·00	0·17	0·121	0·071
[2-^{14}C]caffeic acid	943	2·40	0·39	0·031	0·021
4-Glucosido[2-^{14}C]caffeic acid	360	2·79	0·51	0·215	0·051

Synthesis of chlorogenic acid was observed in leaves of sunflower, chicory and potato, after [2-^{14}C]cinnamic acid was fed. Other metabolites isolated from fed tobacco were also detected in these plants. The results of these feedings are indicated qualitatively in Table 4.

Once formed, chlorogenic acid was metabolized further very slowly in tobacco leaves. When labelled cinnamic acid was allowed to metabolize up to 32 hr in tobacco leaf disks, butanol–acetic acid–water chromatograms showed a slight decline in radioactivity associated with chlorogenic acid (R_f 0·68) and a corresponding appearance of ^{14}C in a second component (R_f 0·57) initially believed to be neochlorogenic acid. Further chromatography showed the label to be associated instead with 6-methoxy-7-glucosyloxycoumarin (scopolin). To confirm these results, labelled chlorogenic acid was prepared and fed to mature tobacco leaves and to young shoots for metabolic periods up to 72 hr. Again scopolin was the main soluble metabolite; caffeic acid and caffeoylglucose were also faintly labelled. No labelled feruloylquinic acids were detected and no migration of the caffeoyl group about the quinic acid moiety occurred. Most of the chlorogenic acid was unmetabolized after 3 days. Table 5 summarizes these results. The rapid initial incorporation of label into fibre must have occurred before the labelled acid reached the pool site, for little subsequent metabolism took place in this direction.

TABLE 2. DISTRIBUTION OF METABOLITES OF LABELLED SUBSTRATES BY TOBACCO LEAF DISKS AFTER 4 hr OF METABOLISM. MAIN FIGURES REPRESENT INCORPORATION OF LABEL AS PER CENT OF SUBSTRATE UPTAKE, THE VALUES IN PARENTHESIS DENOTE THE INCORPORATION OF LABEL AS PER CENT OF TOTAL METABOLITE ACTIVITY

Compound fed	Labelled metabolites						
	Cinnamoyl glucose	*p*-Coumaric acid	*p*-Coumaroyl glucose	*p*-Coumaroyl quinic	Caffeic acid	Caffeoyl glucose	Caffeoyl quinic
[2-^{14}C]Cinnamic acid							
(*a*)	2·8 (5·8)	0·5 (1·1)	trace	trace	+	–	41·0 (84·)
(*b*)	3·4 (13·)	3·1 (13·)	trace	trace	+	–	14·6 (60·)
[2-^{14}C]*p*-Coumaric acid			4·9 (12·)	1·3 (3·0)	2·4 (5·9)	0·5 (1·1)	30·5 (73·)
[2^{14}C]Caffeic acid	—	—	—	—		$0{\cdot}1_5$ (3·1)	2·8 (60·)
4-Glucosido[2-^{14}C]caffeic acid	—	—	—	—	0·9 (9·)	6·3 (63·)	1·7 (17·)

TABLE 3. METABOLISM OF CINNAMIC ACID IN TOBACCO LEAVES IN PRESENCE OF EXCESS *p*-COUMARIC AND CAFFEIC ACIDS (3 hr)

[2-^{14}C]Cinnamic acid fed (μc)	Trapping compound	Labelled metabolites (mμc)					
		p-Coumaric acid	*p*-Coumaroyl glucose	*p*-Coumaroyl quinic	Caffeic acid	Caffeoyl glucose	Caffeoyl quinic
6·38 (1060·)	*p*-Coumaric acid	2234	71	trace	24	trace	20
2·23 (1060·)	Caffeic acid	11	189	8	11 (3·5)	38 (2·8)	405 (9·1)

The values in parentheses are specific activities in μc/mmole.

TABLE 4. LABELLED METABOLITES OF [2-^{14}C]CINNAMIC ACID FROM LEAVES OF VARIOUS PLANTS AFTER 4 hr OF METABOLISM

Plant	Compounds labelled						
	Cinnamoyl glucose	*p*-Coumaric acid	*p*-Coumaroyl glucose	*p*-Coumaroyl quinic	Caffeic acid	Caffeoyl glucose	Caffeoyl quinic (%)
Tobacco	+	+	+	+	+?	−	+++ (56)
Chicory	+	+	++	+	+?	−	+++ (41)
Potato	+	+	+	+	−?	−	++ (12)
Sunflower	+	+	+	++	−?	−	++ (8)

Relative intensity of radioactivity is indicated by crosses. Residual cinnamic acid was present in all cases. The values in parentheses represent the per cent of radioactivity found in chlorogenic acid derived from the substrate.

TABLE 5. METABOLISM OF LABELLED CHLOROGENIC ACID IN SHOOTS AND LEAVES OF TOBACCO

Plant part fed	μc fed	Metabolism time (hr)	Ethanol solubles (μc)	Fibre (μc)
Mature leaves	2·69	24	2·08	0·56
	2·69	72	2·11	0·55
3-month shoots	1·05	6	0·52	0·48
	1·15	24	0·54	0·54
	1·15	48	0·72	0·38

In every case, unchanged material was the only major labelled component of the soluble fraction; some ^{14}C was found in caffeic acid, caffeoylglucose and scopolin.

DISCUSSION

The ability of leaves of plants of several genera to convert cinnamic acid to similar mixtures of products in similar time intervals shows that a common metabolic system is involved in the plants studied. This suggests that similar pathways operate in the formation of chlorogenic acid, which occurs in all species used. The metabolic period of 4 hr used in these experiments was a good deal shorter than the intervals employed in most previous work, yet the conversion of tracer cinnamic acid seemed to be nearly complete in this time.

Also, the high percentage incorporations into chlorogenic acid obtained here in detached leaves have not been reported in other systems. The use of even shorter metabolic times did not result in better detection of intermediates, but rather in lower incorporations into chlorogenic acid together with greater residues of unmetabolized substrate. Thus the intermediates appear to be transient.

The incorporations of label from various substrates definitely demonstrated that caffeic acid as well as *p*-coumaric acid gives rise to chlorogenic acid. The use of 4-glucosidocaffeic acid was an attempt to introduce caffeic acid in a soluble form inside the tissue by the expected hydrolysis of the glucoside, simulating in this way endogenous caffeic acid. While the

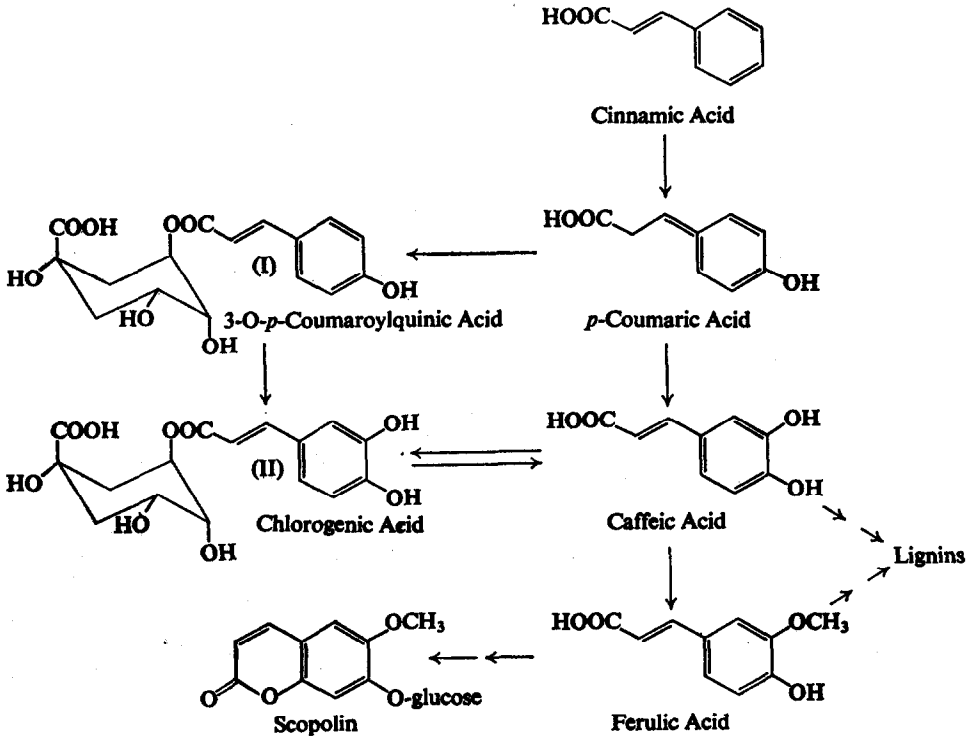

FIG. 1. PROPOSED METABOLIC PATHWAYS CONNECTED WITH CHLOROGENIC ACID IN TOBACCO.

metabolites indicated this was accomplished successfully, the metabolism was not qualitatively different from that of exogenous caffeic acid.

The two trapping experiments provided evidence for a double pathway from *p*-coumaric to chlorogenic acid. Label from [2-^{14}C]cinnamic acid, almost entirely found in the trapping *p*-coumaric acid, indicated that hydroxylation (rather than esterification) was the primary reaction of cinnamic acid. Label from cinnamate was not principally held up by trapping caffeic acid, but appeared in chlorogenic acid. Moreover, in this feeding the specific activity of chlorogenic acid was higher than that of caffeic acid or caffeoylglucose, which could only be so if the major pathway to chlorogenic acid bypassed caffeic acid. The most likely route would seem to involve 3-*O*-*p*-coumaroylquinic acid, as suggested by others, so that the biosynthesis could take place as shown in Fig. 1. This figure also outlines a suggested breakdown route of chlorogenic acid to caffeic acid, ferulic acid, scopolin and lignins. As the

factors balancing the steps of this scheme are not known, one cannot say whether both caffeic acid and *p*-coumaroylquinic acid are chlorogenic acid precursors in all biosynthetic systems, or even in tobacco at all stages of growth. Some other systems are now being studied to clarify this.

The enzymes for quinic acid attachment act on *p*-coumaric, caffeic and ferulic[6] acids. In contrast to enzymes for conversion of cinnamate to *p*-coumarate,[7,8] of *p*-coumarate to caffeate[9] and of caffeate to ferulate,[10] these enzymes have not been studied in cell-free media. That more than one enzyme is involved seems indicated by the disparity in utilization of precursors in formation of chlorogenic and neochlorogenic acids, and by the formation, observed in *Xanthium*,[11] of 3,5-dicaffeoylquinic acid from chlorogenic acid.

EXPERIMENTAL

Plants

Tobacco (*Nicotiana tabacum* L. var. Hicks), sunflower (*Helianthus annuus* L.) and chicory (*Cichorium intybus* L.) were grown in the greenhouse using soilless culture techniques. Potato plants (*Solanum tuberosum* L.) were grown outdoors. Mature leaves of flowering plants were used.

Feeding Techniques

Detached leaves of tobacco, sunflower and potato were fed solutions of labelled compounds in 0·1 N $NaHCO_3$ through the petiole cut ends. Intact chicory leaves were fed by injection into leaf midveins. Leaf disks (dia. 1·3 cm) of tobacco were fed in batches of eight by flotation on radioactive solutions (1·0 ml) in small Petri dishes.

Isolation and Identification of Metabolites

The plant material was boiled, then macerated, in ethanol, and insoluble materials filtered off and washed with hot ethanol. The combined filtrate and washings were evaporated to dryness and extracted with a small volume of hot water. All or part of this water-soluble fraction was spotted on paper and chromatographed first in *n*-butanol:acetic acid:water (BAW) (20:5:11, descending), then in the second direction in benzene:acetic acid:water (BzAW) (125:72:3, descending) to separate aglycones. The low-R_f region containing chlorogenic acid and some other conjugates was then cut away, sewn on to fresh paper and chromatographed in *n*-butanol:pyridine:water (BPW) (14:3:3, descending). Metabolites were identified by R_f values in these and (where necessary) other solvent systems, by observation of fluorescence in u.v. light,[12] and by enzymic hydrolyses with emulsin (glucosides and glucose esters) and pectinase (esters of all kinds). Authentic samples of all the metabolites were available for comparison. Specific activities of metabolites were obtained by eluting the spots from the paper chromatograms into quartz cuvettes, adjusting the volume to 2·0 ml and assaying for mass by absorbance measurements, then counting the entire solution in the scintillation counter.

Radioactive Compounds

[2-^{14}C]Cinnamic acid, 2·0 mc/mmole, was purchased from International Chemical and Nuclear Corp., City of Industry, Calif. [2-^{14}C]*p*-Coumaric acid and [2-^{14}C]caffeic acid, both 943 μc/mmole, were synthesized from [2-^{14}C]malonic acid and the appropriate aldehyde.[13] 4-Glucosido[2-^{14}C]caffeic acid, 360 μc/mmole, was prepared as described earlier.[14] [^{14}C]Chlorogenic acid was prepared biochemically[11] from 34·3 μc [2-^{14}C]cinnamic acid (2·0 mc/mmole) by feeding this to an illuminated tobacco leaf. After 4 hr metabolism the water-soluble fraction (25 μc) was chromatographed on a 2 × 10 cm column of Polyclar AT powder (170 mesh). Elution, begun with water, was changed in steps to 80 per cent methanol over the first 120 ml, by which time several minor active components had emerged. Labelled chlorogenic acid was collected at elution volume

[6] V. C. RUNECKLES, *Can. J. Biochem. Physiol.* **41**, 2259 (1963).
[7] P. M. NAIR and L. C. VINING, *Phytochem.* **4**, 161 (1965).
[8] D. W. RUSSELL and E. E. CONN, *Arch. Biochem. Biophys.* **122**, 256 (1967).
[9] M. SATO, *Phytochem.* **5**, 385 (1966).
[10] B. J. FINKLE and R. F. NELSON, *Biochim. Biophys. Acta* **78**, 747 (1963).
[11] A. O. TAYLOR, *Phytochem.* **7**, 63 (1968).
[12] W. STECK, *Anal. Biochem.* **20**, 553 (1967).
[13] F. VORSATZ, *J. Prakt. Chem.* **145**, 265 (1936).
[14] W. STECK, *Can. J. Biochem.* **45**, 1995 (1967).

150–210 ml; paper chromatography showed it to be the only radioactive component present apart from neochlorogenic acid, from which it was separated on paper in BAW. Yield of [^{14}C]chlorogenic acid was 14·3 μc (radiochemical yield 41 per cent), specific activity about 250 μc/mmole.

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