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Turnover and Metabolism of Chlorogenic Acid in Xanthium Leaves and Potato Tubers[1]

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Summary. The active turnover of chlorogenic acid (3-caffeoylquinic acid[3]), a major phenolic component of *Xanthium* leaves and potato tuber disks, has been demonstrated in these tissues. Pulse-labelling experiments with radioactive L-phenylalanine and *trans*-cinnamic acid as well as direct feeding experiments with chlorogenic acid-^{14}C labelled in the caffeoyl moiety have been employed in the turnover studies. The rate of turnover is calculated to be on the order of 50 to 100 mμmoles per hour per gram fresh weight of tissue.

In *Xanthium* leaves chlorogenic acid is in part converted to an isochlorogenic acid identified by silica gel chromatography as 3,5-dicaffeoylquinic acid. Radioactivity of the caffeoyl moiety of chlorogenic acid is also incorporated into lignin-like insoluble polymers in the leaf. Turnover of chlorogenic acid in tuber tissue is largely accounted for by the incorporation of the caffeoyl moiety into insoluble polymers in the tissue.

The significance of chlorogenic acid turnover is discussed in relation to the perception of the photoperiodic stimulus by leaves and to the possible role of chlorogenic acid in lignin synthesis.

Short term feeding experiments with radioactive L-phenylalanine or *trans*-cinnamic acid indicate that hydroxycinnamic acids and related phenolic compounds show a constant turnover in plant tissues (1, 12). The turnover of these components has been related to their role as intermediates in the formation of lignin polymers (3). Recently El-Basyouni, Neish and Towers (6) described a series of insoluble esters of hydroxycinnamic acids which appear to be very active metabolically. They proposed that such esters are the actual precursors of lignin and soluble hydroxycinnamic acid derivatives that accumulate in plant cells.

Chlorogenic acid (3-caffeoylquinic acid[3]) is one of the most common soluble phenolic derivatives. The ability of many plant tissues to accumulate this phenolic ester is so great that chlorogenic acid, like lignin, would appear to be an end-product of phenolic biosynthesis. Yet very large fluctuations in chlorogenic acid concentration occur in sunflower seeds during their germination (13) and in expanded tobacco leaves during photoperiodic flower induction (17).

These fluctuations suggest that chlorogenic acid is not just an end-product, but may be capable of an active turnover similar to other hydroxycinnamic acid derivatives. The experiments reported here were designed to test this possibility. The results show that an extensive turnover of chlorogenic acid does indeed occur in living tissue, even in expanded leaves which have attained a constant level of the compound.

Materials and Methods

Plant Material. Xanthium pennsylvanicum (Wall.) was grown from seed during the fall and winter months in a greenhouse. A combination of natural daylight and light from incandescent and fluorescent tubes at night provided continuous illumination. Kennebec potato tubers which had been stored at 4° for several months were also used as a source of material.

Labelled Precursors. L-Phenylalanine-U-^{14}C (375 mc/mmole) and quinine acid-U-^{14}C (5 mc/mmole) were obtained from New England Nuclear Corporation. Generally labelled *trans*-cinnamic acid-

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[3] According to Rule 9 of Carbohydrate Nomenclature (J. Org. Chem. 28: 281–91, 1963) chlorogenic acid is designated as 3-0-caffeoylquinic acid, and the 3,5-dicaffeoyl ester is 3,5-di-0-caffeoylquinic acid. Abbreviated forms have been used in the text to emphasize the relationship between the mono and dicayyeoyl esters of quinic acid.

^{3}H was prepared enzymatically from tritiated L-phenylalanine (800 mc/mmole, Nuclear Chicago Corp.) using a purified phenylalanine ammonia-lyase isolated from potatoes (10). L-Phenylalanine-^{3}H (100 μc) was incubated at room temperature with 25 μliters of enzyme and 0.5 ml of 0.1M borate buffer, pH 8.8. After 2 hours the reaction mixture was acidified and extracted several times with ethyl ether. After washing with water, the ether layer was used as a source of tritiated *trans*-cinnamic acid without further purification. Paper chromatography indicated that cinnamic acid was the only tritiated compound present in this fraction.

Chlorogenic acid-^{14}C labelled in the caffeoyl moiety was isolated from 40 g of potato tuber disks fed L-phenylalanine-U-^{14}C. The disks were exposed to light for 16 hours before adding the radioactive L-phenylalanine (500 cpm/mg fr wt of tissue) in 10 ml of a solution of 0.05 M K quinate. After an additional 8 hours in the light, the disks were ground in ethanol and radioactive chlorogenic acid was isolated by the column chromatographic method of Hanson and Zucker (9). The chlorogenic acid isolated from the column showed no change in specific activity when chromatogrammed on paper in acetic acid or butanol-acetic acid solvents. Alkaline hydrolysis and chromatography of the products indicated that at least 99 % of the radioactivity was in the caffeoyl moiety. This biosynthetic method of preparation yielded about 2 μmoles of chlorogenic acid-^{14}C per g fresh weight of tissue. The specific activity was 36,000 cpm/μmole. Radioactive chlorogenic acid and 3,5-dicaffeoylquinic acid [isochlorogenic acid b (5, 9)] labelled in the caffeoyl moieties were also isolated from *Xanthium* leaf disks fed L-phenylalanine-U-^{14}C for 1 hour. Isolation techniques are described below. Hydrolysis and chromatography of the products indicated that at least 99 % of the label was in the caffeoyl moiety.

Introduction of Radioactive Compounds. In most experiments with *Xanthium,* radioactive substrates were applied in 0.05 ml drops to the upper surface of 1.2 cm disks cut from almost fully expanded leaves which had been dipped in a solution of 0.05 % Tween 80. Within a few minutes the drops spread out and moistened the leaf surface uniformly. After 1 hour the radioactive material not absorbed by the tissue was washed from the surface of the disks by rinsing them 4 or 5 times in large volumes of distilled water and finally by rinsing briefly with 50 % ethanol. After being blotted dry the disks were placed on moist filter paper in a petri dish and maintained in the light (400 ft-c) at 21°. Samples consisting of 5 disks and weighing approximately 120 mg were frozen and lyophyllized at the times indicated in each experiment. When whole leaves on intact plants or excised leaves were used, the radioactive substrates were applied to the leaves as described previously (15).

Potato disks 2 mm thick and 1 cm in diameter were cultured in the light for 16 hours to promote chlorogenic acid biosynthesis (16). Radioactive substrates were then applied to the disks in a manner similar to that used for the leaf disks except that a prior treatment with Tween 80 was not used.

Fractionation and Extraction of Xanthium Leaf Tissue. Samples of 5 leaf disks which froze rapidly upon exposure to high vacuum were lyophyllized, and the dry tissue was ground with chloroform containing 25 % cyclohexane (v/v) in a Tenbroek glass homogenizer to remove lipids. The suspension was centrifuged and the dark green supernatant fluid which contained only a small amount of radioactivity was discarded. The residue was then ground twice with 10 ml portions of absolute methanol. The pale green supernatant fraction collected by centrifugation contained 85 to 90 % of the total extractable chlorogenic and 3, 5-dicaffeoylquinic acids. The residue was then ground in 50 % aqueous methanol, a procedure which removed an additional 10 to 15 % of chlorogenic acid and dicaffeoylquinic acid from the residue. Although the ratio of chlorogenic acid to the dicaffeoylquinic acid was the same in both the absolute methanol and the 50 % methanol extracts, the specific activities of both of the caffeoyl conjugates were significantly lower in the 50 % methanol extract. The difference would suggest that the 2 solvents actually extract different pools of chlorogenic and dicaffeoylquinic acid from the leaf. In the work reported here, only the absolute methanolic fraction containing the bulk of the caffeoylquinic acids has been analyzed. If fresh leaf tissue was ground in methanol directly, leaf oxidases often oxidized the caffeoyl esters before extraction was completed. If oxidation occurred, most of the radioactivity of the extract was associated with compounds which did not migrate on paper with the solvents used. When the tissue was lyophyllized prior to extraction, no radioactive compounds (except phenylalanine when present) remained at the origin of paper chromatograms run in the butyl acetate solvent described below.

The residue remaining after extraction with absolute and 50 % methanol was suspended in 5 ml of 5 N NaOH for 20 minutes to hydrolyze any insoluble phenolic esters similar to those in wheat leaves (6). The hydrolyzate was acidified and extracted several times with ethyl ether. The major radioactive component in the ether phase was identified as caffeic acid on the basis of its absorption spectrum, movement on paper chromatography in benzene-acetic acid-water (125-72-3) and 5 % acetic acid, and reaction with nitrous acid (9). The caffeic acid thus obtained is referred to as insoluble caffeic acid.

The residue remaining in the acidified aqueous phase was centrifuged and washed in water. It was then suspended in 10 ml of 1 N NaOH and heated for 1 hour on a boiling water bath. After cooling, the suspension was diluted to volume and

clarified by centrifugation. The supernatant fraction contained about 85 % of the total radioactivity of the sample. This fraction was used as a measure of the extent of incorporation of radioactivity into protein and lignin fractions of the cell. When *L*-phenylalanine-^{14}C was used as substrate, a good portion of the radioactivity in this fraction could be recovered as phenylalanine after hydrolysis of the protein in 6 N HCl and chromatography in the *n*-butyl alcohol-acetic acid-water solvent.

Isolation and Purification of Chlorogenic Acid and 3,5-Dicaffeoylquinic Acid from Xanthium *Leaf Extracts.* Previously chlorogenic acid and an isochlorogenic acid had been identified as the major phenolic acids in *Xanthium* leaves (15). When methanolic extracts prepared as described above were chromatographed on silica gel columns (9), the presence of both chlorogenic acid and a dicaffeoylquinic acid was confirmed. The single isochlorogenic acid present had an R_{cg} value (9) of almost 1 and was very poorly separated from chlorogenic acid. The position of elution indicates that the *Xanthium* isochlorogenic acid is isochlorogenic acid *b* (9) shown by Corse et al (5) to be 3,5-dicaffeoylquinic acid. If other isomers of chlorogenic acid or the dicaffeoylquinic acid were present, they occurred only in trace amounts. When isochlorogenic acid *b* was purified by paper chromatography as described below and then rechromatographed on a silica gel column, a small amount of isochlorogenic acid *a*, 3,4-dicaffeoylquinic acid (5,9) was found to be present also. This component was considered to be an artifact, produced by migration of the caffeoyl groups during purification (7). Since column chromatography did not separate the mono and dicaffeoyl conjugates completely, the procedure was replaced by a paper chromatographic method. One half of the absolute methanol extract containing about 40 optical density units (9) measured at the absorption maximum of 330 mμ, was concentrated to a small volume in vacuo and applied as a 5 cm streak on Whatman No. 1 paper. The paper was equilibrated overnight with vapors of both the aqueous and organic phase of *n*-butyl acetate: acetic acid: water (4:1:5) (15) and then run ascending in the organic phase, for 4 hours. Before the paper was completely dried, chlorogenic and dicaffeoylquinic acid spots, located by their fluorescence under UV light, were cut out and eluted with 80 % methanol. The concentration of the 2 caffeoyl conjugates was determined from the absorption of the eluates at 330 mμ assuming that the millimolecular extinction coefficients were 20 and 40 for chlorogenic acid and 3,5-dicaffeoylquinic acid respectively (5). The radioactivity in small aliquots of the eluates was counted in a liquid scintillation counter with 60 % efficiency for ^{14}C. In experiments with tritium labelled cinnamic acid, counts were corrected for quenching and adjusted to 100 % counting efficiency. Total activity and specific activities were calculated from these data. In some experiments where only the total radioactivity was measured, the chromatograms were cut into rectangles, 2.5 × 3.5 cm and the radioactivity of each piece determined by placing it directly in a scintillation vial.

A single chromatographic run in the butyl acetate solvent was sufficient to prepare chlorogenic acid and 3,5-dicaffeoylquinic of constant specific activity. Further chromatography in *n*-butyl alcohol-acetic acid-water, 5 % acetic acid or benzene-acetic acid-water did not affect the specific activity of either compound. Chromatography of the eluted caffeoyl esters on silica gel columns also indicated that they contained no contaminating radioactive substances.

In some experiments where radioactive chlorogenic acid and isochlorogenic acid *b* were fed to whole leaves, chromatography in butanol-acetic-water was sufficient to purify the components. In these instances radioactive L-phenylalanine was not a possible contaminant.

Isolation of Chlorogenic Acid from Potato Tuber Disks. Chlorogenic acid was extracted from disks as described previously (9). Each sample of 5 disks, 2 mm thick and 1 cm in diameter weighing 2.0 g, was homogenized in 25 ml of 95 % ethanol. After filtration, the residue was washed several times with alcohol. The combined filtrate and washings were concentrated in vacuo and chromatographed directly on an analytical silica gel column. The washed residue was hydrolyzed in 5 N NaOH and extracted with boiling 1 N NaOH as described above for residues from *Xanthium* leaves.

Results

Turnover Studies with Xanthium *Leaves. Xanthium* leaf disks were allowed to absorb trace quantities (44 mμmoles/g of tissue) of L-phenylalanine-U-^{14}C (375 mc/mmole) for 1 hour at the beginning of the experiment. Usually 50 % or more of the label supplied (2×10^6 cpm per sample of 5 disks) was absorbed by the leaf tissue during this period. The concentration, total radioactivity, and specific activity of chlorogenic acid, 3,5-dicaffeoylquinic acid and the insoluble caffeic acid fraction were assayed in samples of leaf disks taken over a period of 24 hours. Very striking changes occurred in both the total activity (middle graph, fig 1) and the specific activity (upper graph) of the individual caffeoyl conjugates, although little net synthesis was evident during the experiment (lower graph). Chlorogenic acid was labelled rapidly, attaining a high activity within an hour of the start of the experiment. Radioactivity could be detected in the free phenylalanine pool of the tissue for about 4 hours, although the exogenous source had been removed 1 hour after application. The increase in total and specific activity of chlorogenic acid between 1 and 4 hours probably reflects the presence of the endogenous pool of phenylalanine and intermediates formed from it. Thereafter, the total

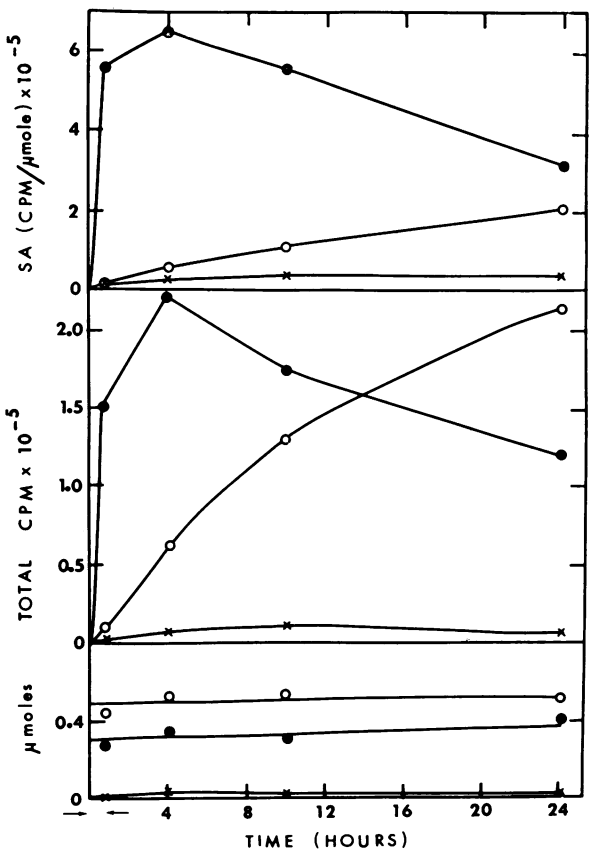

FIG. 1. Turnover of chlorogenic acid in *Xanthium* leaf disks determined by pulse-labelling with radioactive phenylalanine. L-phenylalanine-U-^{14}C (375 μc/mole, 2×10^6 cpm added per sample of 5 disks) was applied to the upper surface of leaf disks 1.2 cm in diameter. After 1 hour, unabsorbed phenylalanine (40 to 50 % of that applied) was washed from the surface of the disks which were then placed on moist filter paper in the light (400 ft-c) at 21°. At times indicated, the following compounds were isolated quantitatively from a sample of 5 disks: 3-caffeoylquinic acid, •—(chlorogenic acid); 3,5-dicaffeoylquinic acid, ○—(isochlorogenic acid b); and insoluble caffeic acid, X—. Data are reported per sample, fresh weight of 120 mg. Arrows along abscissa indicate duration of pulse-labelling.

radioactivity of the chlorogenic acid declines with a loss of about half of the total activity in the fraction by the end of 24 hours. The decrease in radioactivity is direct evidence for a turnover of chlorogenic acid molecules. The turnover is confirmed by the rise and fall in specific activity (upper graph, fig 1) which occurs without a concomitant change in net chlorogenic acid content of the tissue. Similar results were obtained in pulse-labelling experiments with whole leaves attached to plants.

The behavior of 3, 5-dicaffeoylquinic acid is entirely different. Radioactivity appeared very slowly in this compound (middle graph, fig 1) and continued to increase throughout the period of sampling. Between 4 and 24 hours the total radioactivity in the dicaffeoyl ester showed a net increase of 150,000 cpm, while the net loss from the chlorogenic acid fraction was 100,000 cpm. The specific activity of the dicaffeoylquinic acid also increased steadily from 13 cpm per mμmole at 1 hour to 206 cpm per mμmole at 24 hours. Eventually the specific activity of this conjugate surpassed that of chlorogenic acid. The change in activity does not rule out chlorogenic acid as a precursor of 3, 5-dicaffeoylquinic acid. It may simply reflect the very slow turnover of the dicaffeoylquinic acid fraction compared to the rapid metabolism of the chlorogenic acid. Hydrolysis of both fractions indicated that at least 95 % of the label was located in the caffeoyl moieties. The insoluble caffeic acid fraction accumulated radioactivity slowly and showed little if any turnover. This inactivity differentiates it sharply from similar fractions in wheat leaves (6).

In some experiments the combined chlorogenic and dicaffeoyl ester fractions accounted for 80 % of the radioactivity in the soluble fraction. Almost half of the total radioactivity incorporated into the leaf tissue remained in the alcohol insoluble residue after alkaline hydrolysis. Some of the activity appears to be associated with a lignin fraction. If the residue is subjected to alkaline nitrobenzene oxidation (6), a radioactive fraction can be extracted with ethyl ether from the hydrolysate after acidification. The radioactive material has the same R_F as vanillin in alkaline and acid solvents (6) and gives an aldehyde reaction with 2, 4-dinitrophenylhydrazine. Most of the insoluble ^{14}C is associated with phenylalanine residues in protein molecules and can be recovered by paper chromatography as free phenylalanine after acid hydrolysis of the protein. This large reservoir of protein-bound phenylalanine appears to serve as a continuous source of radioactive precursor throughout the time period and complicates the experiment. To obtain a more accurate estimate of the turnover rates and the extent of incorporation of phenolic components into the insoluble residue, another series

Table I. *Incorporation of Radioactivity into the Insoluble Residue of* Xanthium *Leaves*

The insoluble residue was the fraction remaining after extraction of leaf tissue with methanol and hydrolysis with 5 N NaOH. Data are based on 24 hr samples. See legends to figs 1 and 4 for experimental details.

Radioactive substrate	Labelling of insoluble residue as % of total radioactivity absorbed
Cinnamate (4)*	8
L-Phenylalanine (12)	43
3-Caffeoylquinic acid (3) (Caffeoyl moiety labelled)	38
3,5-Dicaffeoylquinic acid (1) (Caffeoyl moiety labelled)	66

* Figures in parentheses indicate the number of experiments upon which average percentages are based.

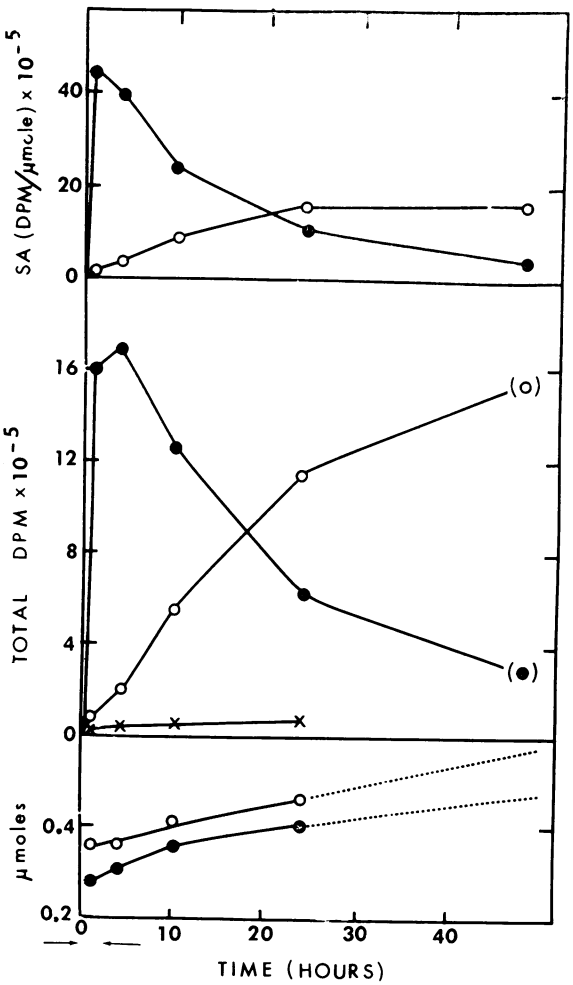

FIG. 2. Turnover of chlorogenic acid in *Xanthium* leaf disks determined by pulse-labelling with radioactive *trans*-cinnamic acid. Generally labelled *trans*-cinnamic acid-^{3}H (800 μc/μmole, 5 $\times$ 10^{6} dpm added per sample of 5 disks) was supplied for 1 hr. Experimental conditions and symbols same as in figure 1. Dotted lines and parentheses indicate estimated value for 48 hr sample which was partially oxidized during preparation.

of pulse-labelling experiments was performed using generally labelled *trans*-cinnamic acid-^{3}H which does not give rise to amino acids.

Tritium labelled cinnamic acid was supplied to disks (800 μc/μmole; 5 $\times$ 10^{6} dpm per sample of 5 disks), and the experiment performed as above. The results (fig 2) confirm those obtained with L-phenylalanine-^{14}C except that much less radioactivity was incorporated into the insoluble residue (table I). As anticipated, the changes in the chlorogenic acid fraction were more dramatic than those obtained with L-phenylalanine. A semi-log plot of the decrease in total activity of chlorogenic acid with time (fig 3) indicates that the decay follows first order kinetics. The half-life of chlorogenic acid calculated from this plot is 14 hours. A similar calculation of the half-life of chlorogenic acid in the L-phenylalanine experiment gives a value of 20 hours. The longer half-life obtained when L-phenylalanine is used as a precursor supports the suggestion that breakdown of proteins in the tissue provides a source of precursor for chlorogenic acid biosynthesis. During preparation of the 48 hour tritium-labelled sample, some of the chlorogenic acid and dicaffeoylquinic acid were oxidized with a consequent loss of radioactivity from the soluble fraction. Abnormally high labelling of the insoluble caffeic acid fraction and the residue were observed in this sample. Although determinations of specific activity were not affected by the oxidations, it was necessary to calculate the total activities from estimates of the concentrations of chlorogenic acid and the dicaffeoyl conjugate shown in the lower graph of figure 2. Assuming the estimates to be accurate, the data indicate that a total of 1.4 $\times$ 10^{6} dpm were lost from the chlorogenic acid fraction between 4 and 48 hours while the total radioactivity of the dicaffeoylquinic acid increased by 1.3 $\times$ 10^{6} dpm during this time. Although the similarity in magnitude of loss of radioactivity from 3-caffeoylquinic acid and increase in activity of the 3, 5-dicaffeoylquinic acid fraction may be fortuitous, it does suggest that the monocaffeoyl ester is a precursor of the dicaffeoyl ester.

In order to investigate directly the relationship between the mono and dicaffeoyl conjugate, a series of feeding experiments were undertaken with chlorogenic acid-^{14}C and 3, 5-dicaffeoylquinic acid-^{14}C, both labelled in the caffeoyl moieties. The esters were applied individually to whole, excised leaves

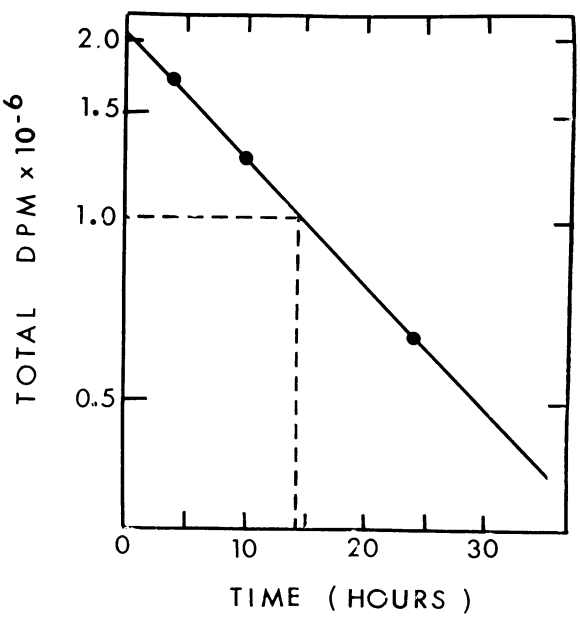

FIG. 3. Rate of turnover of chlorogenic acid in *Xanthium* leaf disks calculated from semi-log plot of data in figure 2. Dotted lines indicate graphic estimation of half-life of chlorogenic acid (14 hrs).

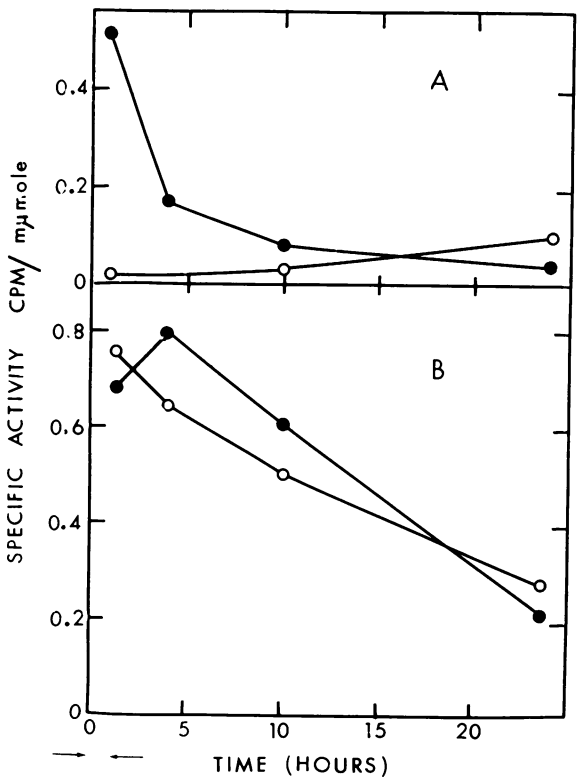

FIG. 4. Metabolism of caffeoyl esters fed to *Xanthium* leaves. A) 3-caffeoylquinic acid-^{14}C labelled in the caffeoyl moiety (456 cpm/mμmole, 90 × 10^3 cpm added) was applied to the upper surface of an excised leaf. After 1 hour the unabsorbed chlorogenic acid was washed from the surface, and the leaf was then maintained on moist filter paper in a petri dish in the light (400 ft-c) at 21°. At the times indicated a sample of 5 disks 1.2 cm in diameter was punched from the leaf and analyzed for chlorogenic acid (•—) and 3,5-dicaffeoylquinic acid (○—). B) 3,5-Dicaffeoylquinic acid-^{14}C labelled in 1 or both caffeoyl moieties (106 cpm/mμmole, 92.5 × 10^6 cpm added) was applied to the surface of an excised leaf, and the leaf was treated as above. Arrows along the abscissa indication time during which chlorogenic acid-^{14}C (A) or 3,5-dicaffeoylquinic acid-^{14}C (B) was applied to the leaf surfaces.

for 1 hour and then washed off. Samples of disks were then punched from the leaves at appropriate intervals. Figure 4 shows the changes in specific activity of the chlorogenic acid and the 3, 5-dicaffeoyl ester fractions in samples of disks taken from leaves supplied with either chlorogenic acid-^{14}C (fig 4A) or 3, 5-dicaffeoylquinic acid-^{14}C (fig 4B).

Exogenously supplied chlorogenic acid which penetrated into the leaf behaved in a manner similar to that of endogenously formed chlorogenic acid. In figure 4A we find a rapid loss of radioactivity from the chlorogenic acid fraction indicating an active turnover of the phenolic ester. A slow labelling of the isochlorogenic acid fraction occurs similar to that observed when radioactive L-phenylalanine or cinnamic acid are used as substrates. Very little of the caffeoyl conjugates penetrated into the leaves during the short pulse feeding experiments. The total activity of the fractions was of the order of only 10 to 1000 cpm. However, the results were essentially confirmed by other experiments in which chlorogenic acid was fed to both young and old leaves for periods of 16 to 28 hours (table II). In these experiments where substantially higher incorporation occurred, radioactivity always disappeared from the chlorogenic acid fraction and appeared slowly in the dicaffeoylquinic acid fraction. Hydrolysis of the recovered fractions indicated that at least 90 % of the activity was associated with the caffeoyl moieties. These data provide additional evidence that the caffeoyl moiety of chlorogenic acid is a precursor to at least 1 of the caffeoyl moieties of isochlorogenic acid.

Table II. *Conversion of 3-Caffeoylquinic Acid-^{14}C (Chlorogenic Acid) to 3,5-Dicaffeoylquinic Acid (Isochlorogenic Acid b) in* Xanthium *Leaves*

Chlorogenic acid-^{14}C labelled in the caffeoyl moiety (1 μmole containing 36,000 cpm) was supplied for 28 hours in the dark to a young leaf and to a fully expanded leaf each attached to separate plants. Both leaves were then washed thoroughly and extracted with methanol. The alcoholic extract of the young leaf and the expanded leaf contained 11,000 and 8 000 cpm respectively. At least 95 % of the radioactivity in the 2 esters isolated was associated with the caffeoyl moieties.

Leaf size	Compound isolated	
	3-caffeoylquinic acid	3,5-dicaffeoyl quinic acid
	cpm/leaf	
one-third expanded	1630	1080
fully expanded	1285	1010

The feeding experiment with radioactive dicaffeoylquinic acid shown in figure 4B indicates an additional relationship between the 2 caffeoyl conjugates. Within an hour, radioactivity from the 3, 5-diester can be detected in the caffeoyl moiety of chlorogenic acid. Apparently the mono and dicaffeoyl conjugates are interconvertable. The subsequent disappearance of label from the chlorogenic acid is consistent with the already established view that it turns over rapidly in the leaf tissue. Since little change in the total amount of the dicaffeoyl ester occurred during the experiment, the decrease in specific activity suggests that the diester fraction also turns over.

Some of the long term feeding experiments conducted with whole plants afforded an opportunity to investigate the possible translocation of chlorogenic acid from leaves to the growing point. When chlorogenic acid-^{14}C labelled in the caffeoyl moiety was applied to leaves of intact plants, radioactivity was occasionally found in the stem tip section. But,

in no instance did the total activity outside of the leaf exceed 10 % of that remaining in the leaf. Translocation, therefore is not considered to be an important factor in the metabolism of chlorogenic acid in *Xanthium* leaves.

One major difference between feeding experiments with cinnamic acid and with chlorogenic acid is the extent of incorporation of radioactivity into the alcohol insoluble fraction of the leaf. Little activity was incorporated into the insoluble fraction when radioactive cinnamic acid was used as substrate. The amount of activity accumulating in the fraction usually did not exceed 10 % of the total activity incorporated. On the other hand, addition of chlorogenic acid to the leaf produced a rapid labelling of the alcohol insoluble residue. As much as 50 % of the total activity in the tissue could be isolated in the insoluble fraction within an hour of feeding the conjugates. A comparison of incorporation of activity into the insoluble fraction from various precursors is shown in table I.

The heavy labelling obtained with phenylalanine comes more from protein bound, radioactive phenylalanine, than from phenolic polymers such as lignins. The small incorporation from cinnamic acid which increased with time probably represents a truer picture of lignin synthesis. Rapid incorporation of radioactivity from the caffeoyl moiety of chlorogenic acid suggests an oxidation and polymerization of the products as the caffeoyl conjugate penetrates the leaf. However, once chlorogenic acid has entered the cell, it appears to behave as chlorogenic acid synthesized endogenously.

Turnover Studies with Potato Tuber Disks. Unlike the leaf tissues studied, disks cut from the pulp of the potato tuber initially contain little chlorogenic acid. They will accumulate large quantities of the conjugate if exposed to light for 16 to 24 hours. If radioactive L-phenylalanine is supplied to freshly cut disks and then removed after a short period of time, the total radioactivity in the chlorogenic acid fraction will increase over a 24 hour period roughly in proportion to the amount of chlorogenic acid accumulating in the tissue. Under these conditions, turnover, if it exists, is masked by the extensive synthesis of the compound. However, if the tissue is first allowed to initiate phenolic biosynthesis and accumulate chlorogenic acid before supplying a pulse of labelled phenylalanine, a rapid turnover of chlorogenic acid can be demonstrated. In the experiments described below, disks of tuber tissue were cultured 16 hours in the light to stimulate phenolic biosynthesis and increase the level of chlorogenic acid in the tissue, before a trace quantity of L-phenylalanine-U-^{14}C (1.2×10^6 cpm/g fr wt) was added to the tissue. After 1 hour, the unabsorbed phenylalanine was washed off the tissue. The disks were then transferred to darkness in order to decrease the rate of chlorogenic acid synthesis and thus prevent a masking of turnover by continued incorporation of label from endogenous

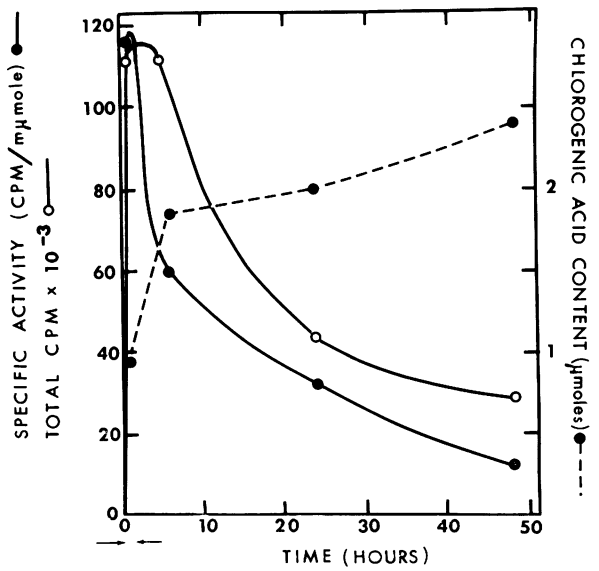

FIG. 5. Turnover of chlorogenic acid in potato tuber disks determined by pulse-labelling with radioactive phenylalanine. L-phenylalanine-U-^{14}C (375 μc/μmole, 2.5×10^6 cpm added per sample of 5 disks) was applied to the surface of disks 1 cm in diameter and 2 mm thick which had been previously maintained moistened for 16 hours in light (400 ft-c) at 21°. After 1 hour, phenylalanine not absorbed by the disks (approximately one-third of that added) was washed from the surface, and the disks were subsequently kept moistened in petri dishes in darkness. Samples of 5 disks originally weighing 2.0 g were withdrawn at times indicated and analyzed for chlorogenic acid. Arrows along abscissa indicate duration of pulse-labelling. ●—, specific activity of chlorogenic acid isolated; ○—, total activity of chlorogenic acid per sample of 5 disks; ●- - -, Chlorogenic acid content, μmoles per 5 disks.

pools of radioactive phenylalanine. Figure 5 shows the results of such an experiment. Radioactivity from L-phenylalanine was rapidly incorporated into the caffeoyl moiety of chlorogenic acid. Upon removal of exogenous phenylalanine, the radioactivity of the endogenous pool of free phenylalanine fell rapidly and little ^{14}C could be detected in this fraction after 6 hours. By this time radioactivity began to disappear from the chlorogenic acid fraction. After 48 hours, 80 % of the label had been lost from chlorogenic acid. Assuming that the 2.5-fold increase in chlorogenic acid content during the experiment represented synthesis from unlabelled precursors, a corresponding drop in the specific activity of chlorogenic acid would be expected. The actual change observed was much greater, i.e., a 10-fold decrease in specific activity. The decrease in both total and specific activity of chlorogenic acid under these conditions indicates that it turns over rapidly in potato tuber disks as well as in *Xanthium* leaves. The rate of turnover is similar to that in the leaf tissues.

No corresponding increase in radioactivity of

Table III. *Distribution of Radioactivity Between Fractions of Potato Tuber Disks Supplied* L-*Phenylalanine-U-*^{14}C

Disks of potato tuber were treated as described in figure 5. At times indicated, a sample of 5 disks corresponding to an original fresh weight of 2.0 g was homogenized in 95 % ethanol, and an aliquot of the homogenate was counted to determine total radioactivity. After filtration, an aliquot of the filtrate was used to measure activity of soluble components. Chlorogenic acid was isolated from the filtrate by chromatography on silica gel (9).

Time after Removal of Phenylalanine-^{14}C	Total	Alcohol soluble components	Alcohol insoluble residue*	Chlorogenic acid
			cpm per mg fr wt	
1 hr	903	260	643	56
48 hrs	910	191	719	14
Difference		—69	+79	—42

* Estimated by difference.

other hydroxycinnamic acids occurs in tuber disks. However, the alcohol insoluble fraction of the tissue increases in activity. Table III shows the relationship between the change in radioactivity of the alcohol soluble fraction, the alcohol insoluble fraction, and chlorogenic acid which initially contains about 20 % of the radioactivity of the alcoholic extract. The data in this table suggest that the turnover of chlorogenic acid represents its conversion to insoluble polymers, particularly since ^{14}C does not accumulate in other soluble phenolic compounds.

Discussion

The present studies show that chlorogenic acid, a major phenolic ester accumulating in *Xanthium* leaves and potato tuber disks, exists in a dynamic state of turnover. The concentration of chlorogenic acid observed in mature leaf tissue represents a steady state equilibrium in which the rate of synthesis balances the rate of utilization. If we assume that the half-life of chlorogenic acid in two-third expanded *Xanthium* leaves is 14 hours (fig 3) and that the steady state concentration in the same leaves is about 3 μmoles per g fresh weight (fig 1, 2), then the rate of turnover is approximately 100 mμmoles per hour per g of tissue. Similar calculations for tuber disks suggest a turnover rate of about 50 mμmoles of chlorogenic acid per hour per g fresh weight. These figures refer to the rate of utilization of the caffeoyl moiety; the ester linkage may be far more labile.

The metabolism of chlorogenic acid (3-caffeoylquinic acid) in *Xanthium* leaves represents in part the conversion of this monocaffeoyl ester to the dicaffeoyl ester, 3, 5-dicaffeoylquinic acid. Direct feeding experiments with radioactive chlorogenic acid demonstrated that at least 1 of the caffeoyl moieties of the dicaffeoyl ester is derived from chlorogenic acid. Esterification or transesterification of the 5 hydroxyl of the quinic moiety of chlorogenic acid by a caffeoyl group would produce a direct transformation and allow the conservation of the ester bond at the 3 position of the quinic moiety. The fact that chlorogenic acid becomes labelled rapidly when radioactive 3, 5-dicaffeoylquinic acid is added to the leaves suggests that an interconversion of the mono and dicaffeoylquinic acids occur. However, at present we do not know the source of the second caffeoyl moiety of 3, 5-dicaffeoylquinic acid. Nor can we eliminate the possibility that chlorogenic acid is hydrolyzed to release free caffeic acid which serves as the source of both caffeoyl moieties in the dicaffeoyl ester.

The turnover rate of the 3, 5-diester is much less than that of chlorogenic acid. The initial rate of incorporation of radioactivity from L-phenylalanine or cinnamic acid into the single caffeoyl moiety of chlorogenic acid is about 20 times greater than the rate of labelling of the 2 caffeoyl groups in the dicaffeoylquinic acid. If all of the chlorogenic acid turnover merely represented its conversion to 3, 5-dicaffeoylquinic acid, then a very much greater quantity of the dicaffeoyl ester should accumulate in the leaf compared to chlorogenic acid. The actual analyses reported here indicated that the concentration of the dicaffeoylquinic acid is only slightly greater than that of chlorogenic acid, even in mature leaves. Consequently, the turnover of chlorogenic acid appears to involve other metabolic conversions as well. Free caffeic acid is a lignin precursor (3). Utilization of the caffeoyl group of chlorogenic acid for the synthesis of phenolic polymers such as lignin is 1 possibility that must be entertained.

The extent of the overall incorporation of radioactivity from cinnamic acid into the insoluble fraction of the leaf provides an estimate of the upper limit of lignin synthesis. In *Xanthium* leaves this value represents an incorporation of about 10 % of the total absorbed activity over a period of 24 hours. A similar percentage of incorporation of absorbed cinnamic acid into the insoluble residue of apple leaves has been reported (1). In the experiment described in figure 2 the insoluble residue contained 24×10^5 dpm per g of tissue after 24 hours. The average specific activity of chlorogenic acid during this period was about 24×10^5 dpm per μmole. If all of the insoluble activity were derived from the

labelled caffeoyl moiety of chlorogenic acid, then approximately 1 μmole of caffeic acid equivalent to 40 % of the chlorogenic acid turned over would have been utilized for synthesis of insoluble polymers. On this basis, a leaf 30 days old would be expected to have a lignin content of approximately 0.5 % of the fresh weight. This figure is not an unreasonable one.

In contrast to the slow rate of incorporation of radioactivity into insoluble polymers when radioactive cinnamic acid is applied to leaves, a very rapid incorporation of radioactivity occurs when chlorogenic acid labelled in the caffeoyl moiety is supplied exogenously. This difference in behavior of exogenous chlorogenic acid and chlorogenic acid formed endogenously from labelled cinnamic acid suggests a difference in accessibility to sites of polymerization. Lignin synthesis is thought to occur in the primary and secondary cell walls (2). Molecules of chlorogenic acid penetrating through the cell walls from the external surface of the leaf might encounter such sites more readily than endogenously formed molecules which would have to pass through at least 1 cytoplasmic membrane. Stafford has reported that cell wall fractions of *Phleum* will synthesize lignin from sucrose and hydrogen peroxide (14). Although the possibility that her cell wall fractions were contaminated with whole cells was not excluded, the data taken at face value indicate that the cell walls may contain enzymes capable of converting possible intermediates such as chlorogenic acid into lignin. The fact that a vanillin-like, radioactive compound can be obtained from nitrobenzene oxidation of the insoluble residue of leaf tissue supplied with radioactive chlorogenic acid also suggests that the caffeoyl moiety serves as an intermediate in lignification.

Chlorogenic acid is the only major phenolic compound in potato tuber disks (9). Consequently, it cannot be conserved during turnover by conversion to phenolic derivatives as in *Xanthium* leaves. Rather, turnover of chlorogenic acid in tuber disks results in a loss of radioactivity from the soluble fraction and an increase in labelling of the alcohol insoluble residue. That is, the caffeoyl moiety of chlorogenic acid is converted into insoluble polymers. This type of reaction involving chlorogenic acid molecules formed endogenously, should not be confused with reactions which result from the release of oxidative enzymes and phenolic substrates from broken cells on the surface of cut disks. The rate of incorporation of leucine into protein of tuber disks increases greatly during 24 hours of culture (4). It is possible that the rate of lignification of tuber disks is much greater than that within the whole tuber. Consequently, the turnover of chlorogenic acid may be exaggerated under the conditions of the experiment. However, such conditions might be encountered in the case of fungal infections or wounding of the tissue by other means.

A rapid turnover of insoluble hydroxycinnamoyl esters has been demonstrated in wheat leaves (6). These compounds have been implicated as a precursor to lignins and to soluble esters such as chlorogenic acid. A search for similar fractions in *Xanthium* leaves and potato disks has failed to reveal such metabolically active components. *Xanthium* leaves do contain relatively large quantities of an alcohol insoluble caffeoyl ester. However, this fraction turns over very slowly if at all. Almost no radioactivity is associated with the trace quantities of alkali labile, insoluble components in potato residues. These results agree with the conclusions of Hanson (8).

Avadhani and Towers (1) have failed to find an extensive turnover of chlorogenic acid in mature apple leaf disks. However, in their experiments, tissue was exposed to radioactive L-phenylalanine for 16 hours prior to the study of turnover. It is possible that the only radioactive chlorogenic acid remaining after a 16 hour feeding experiment was that stored at an inactive site such as the cell vacuole. It is also possible that no turnover exists in the mature leaf tissue. Examination of both young and old *Xanthium* leaf tissue indicates that an active turnover of chlorogenic acid occurs regardless of age in this tissue (manuscript in preparation).

The fact that chlorogenic acid shows an extensive turnover in expanded leaves adds considerable interest to the possible metabolic role of this ubiquitous compound in plants. In *Nicotiana*, photoperiodic flower induction affects the metabolism of chlorogenic acid and its related isomers in leaves. Very large fluctuations in chlorogenic acid concentrations of expanded leaves occur during photoperiodic induction of both short and long day species (17). This photoperiodic effect could involve changes in the rate of turnover of chlorogenic acid and its isomers. Studies are now in progress to determine the relationship between photoperiodic induction and the turnover of chlorogenic acid in leaf tissue.

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