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THE DISTRIBUTION AND TURNOVER RATE OF SOLUBLE AND INSOLUBLE CAFFEOYL ESTERS IN *XANTHIUM*

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Abstract—Chlorogenic acid labelled in the caffeoyl moiety from *trans*-(U-^{14}C)cinnamic acid has a half-life of 9 hr in young leaves, 24 hr in mature leaves, 19 hr in young stem sections and 14 hr in roots of *Xanthium*. As label is lost from chlorogenic acid, activity appears largely in 3,5-dicaffeoylquinic acid and insoluble residues. The rate of gain or loss of activity of these fractions differs in the various tissues, but some generalities can be made. During the 2 hr immediately following tracer application (applied for 1 hr) most of the label appears in insoluble residues. In a subsequent 20 hr period there is a slower though continued incorporation into this fraction in young leaves and roots. The rate of loss of activity from chlorogenic acid during the 2–22 hr after application of the tracer suggests a first order reaction of utilization. Most of the label appears in 3,5-dicaffeoylquinic acid during this time. From a consideration of these results and others obtained by feeding caffeoyl or quinic labelled chlorogenic acid to *Xanthium* leaves, it appears that the enzymes for lignin synthesis may be in the cell wall. As chlorogenic acid enters the primary cell wall or passes through the plasmalemma, many of the caffeoyl and quinic moieties arising by de-esterification are utilized in the synthesis of insoluble polymers, presumably lignin. The remainder migrates into the cytoplasm where a predominant chlorogenic to 3,5-dicaffeoylquinic acid conversion is observed. Insoluble caffeoyl esters which occur in *Xanthium*, especially in the roots and young leaves, are labelled very slowly and appear to play no part in the interrelations discussed.

INTRODUCTION

EL-BASYOUNI, Neish and Towers have recently described a series of alcohol-insoluble hydroxycinnamic acid esters in wheat and barley[1,2] which appear to be metabolically active because of their rapid and extensive turnover. They propose that such esters are the actual precursors of lignin and of the soluble hydroxycinnamic acid derivatives that accumulate in plant cells. In *Xanthium*[3] and potato,[3,4] alcohol-insoluble compounds of this type are present in small or trace amounts, accumulate little activity from labelled L-phenylalanine or cinnamic acid and turn over slowly, if at all. In contrast to this chlorogenic acid (3-caffeoylquinic acid) becomes rapidly and extensively labelled in the caffeoyl moiety from L-phenylalanine or cinnamic acid, and has a half life of 14 hr in discs punched from two-thirds expanded *Xanthium* leaves, and 20 hr in potato tuber discs.[3] In *Xanthium*, a large proportion (93 per cent) of the label disappearing from chlorogenic acid appeared in 3,5-dicaffeoylquinic acid during a 44 hr period, yet during the same period there was a significant increase in the labelling of insoluble residues, presumably lignin.[3] From these data on leaves of one developmental

[1] SAID Z. EL-BASYOUNI, A. C. NEISH and G. H. N. TOWERS, *Phytochem.* **3**, 627 (1964).
[2] SAID Z. EL-BASYOUNI and A. C. NEISH, *Phytochem.* **5**, 683 (1966).
[3] A. O. TAYLOR and MILTON ZUCKER, *Plant Physiol.* **41**, 1350 (1966).
[4] K. R. HANSON, *Phytochem.* **5**, 491 (1966).

age, calculations indicate that 40 per cent of the chlorogenic acid turnover would be needed to account for the amount of lignin labelled. These results are not compatible if all the label is coming from chlorogenic acid.

In this work the turnover of soluble and insoluble caffeoyl esters in leaves of differing developmental age and in stems and roots of *X. pensylvanicum* have been investigated in an attempt to clarify these inconsistencies. Labelling of the caffeoyl esters from $^{14}CO_2$ has been compared with the pattern of incorporation obtained using labelled *trans*-cinnamate to indicate whether cinnamic acid utilization occurs via the normal obligatory biosynthetic pathway of these esters. In addition the incorporation of activity into alcohol-insoluble components from chlorogenic acid labelled predominantly in the caffeoyl or the quinic moiety have been compared to learn more of the reactions involved.

RESULTS

Turnover Studies using Trans-(U-^{14}C) Cinnamic Acid

Rather than attempting to follow changes during the development of a single leaf, discs were taken from leaves of differing size. These discs, stem sections and roots were obtained from *Xanthium* plants raised in continuous light to a stage shown in Fig. 1. At this age the sixth leaf has just become fully expanded (78 cm^2) while the two leaves immediately above it are 24 and 5 cm^2 in area respectively. These three leaves are reasonably comparable in that they expand at the same rate to a similar final size and are equivalent in their photoperiodic responsiveness.[5] A leaf 5 cm^2 in area will get to the L24 stage in 2 days and become fully expanded (78 cm^2) in a further 8–10 days.[6]

Tissues were fed *trans*-(U-^{14}C) cinnamic acid 350 mc/mM ($4{\cdot}06 \times 10^6$ dmp/treatment) for 1 hr, then allowed to metabolize in the light whilst samples were taken over a 21 hr period. No significant net synthesis of the soluble caffeoyl esters or of insoluble caffeoyl esters was observed during this period in any of the tissues. Actual amounts of the soluble and insoluble esters present in the various plant parts are shown in Table 1. As a leaf matures, the ratio of 3,5-dicaffeoylquinic acid to chlorogenic acid and the concentration of these acids on a dry weight basis fall markedly. Young stem sections have high soluble ester levels, roughly

TABLE 1. CONCENTRATIONS OF CHLOROGENIC ACID, 3,5-DICAFFEOYLQUINIC ACID AND INSOLUBLE CAFFEIC ACID IN LEAVES OF DIFFERING AGES AND IN YOUNG STEMS AND ROOTS OF *Xanthium*

Tissue	Chlorogenic acid	3,5-Dicaffeoyl-quinic acid (μM/g dry weight)	Insoluble caffeic acid
Leaf 5*	38·3 (1·08)†	91·3 (2·57)	18·5 (0·52)
Leaf 24	26·1 (2·89)	34·3 (3·79)	16·1 (1·79)
Leaf 78	21·3 (6·14)	19·6 (5·65)	3·62 (1·04)
Leaf senescing	24·8	15·2	1·01
Stem	43·4	76·6	2·32
Root	12·3	14·7	12·3

* Numbers refer to the area of the leaf (cm^2).
† Figures in parenthesis indicate total amount (μM) in a leaf of this area.

[5] A. K. KHUDAIRI and K. C. HAMNER, *Plant Physiol.* **29**, 251 (1954).
[6] R. MAKSYMOWYCH, *Am. J. Botany* **49**, 7 (1962).

equal to those in young leaves, though insoluble esters are much lower than in young leaves. Roots contain lower levels of soluble esters than any of the aerial plant parts analyzed, yet reasonably high levels of insoluble esters.

During the 10–12-day expansion of a leaf from 5 cm^2 to 78 $cm,^2$ the amount of chlorogenic acid (per leaf) increases 6 fold (5·06 μM actual increase), while the 3-5-dicaffeoylquinic acid level increases 2–3 fold (3·08 μM actual increase). Insoluble caffeoyl esters increase substantially over the same 10–12-day period, but remain at approximately one tenth the combined μ molar concentration of the soluble esters.

Chlorogenic acid and 3,5-dicaffeoylquinic acid are the main labelled components in the alcohol extract. Most of the remainder appears in a compound(s) remaining at the origin of the *n*-butyl acetate-acetic acid-water developed chromatograms (especially in young leaves) but the degree of labelling remains reasonably constant over the period of the experiment. This labelling does not appear to be the result of an extraction induced oxidation of caffeoylquinic acids.[3] Little activity appears in these compounds in extracts of young stems which contain large concentrations of labelled caffeoyl esters and also high oxidase levels as judged by their rate of browning following bruising.

TABLE 2. SPECIFIC ACTIVITIES OF CHLOROGENIC ACID AND 3,5-DICAFFEOYLQUINIC ACID AT VARIOUS TIMES AFTER CESSATION OF FEEDING THE LABELLED PRECURSOR *trans*-(U-^{14}C) *cinnamic acid*

Hours after removal of precursor*	Specific activity (dpm/μM × 10^{-3})†											
	Leaf 5‡		Leaf 24		Leaf 78		Leaf senescing		Stem		Root	
	CA	DCA	CA	DCA	CA	DCA	CA	DCA	CA	DCA	CA	DCA
1	63·5	9·4	110	10·5	259	11·7	391	13·7	98·1	3·5	197	18·2
3	48·5	12·4	85·6	14·5	230	14·1	364	19·8	87·9	9·3	155	21·5
10	32·3	21·3	63·9	28·8	201	29·2	302	40·1	70·4	18·5	86	26·6
21	15·6	34·1	31·3	48·8	142	53·4	210	78·7	49·5	38·4	82	59·5
½ life of CA§	9 hr		12.5 hr		24 hr		23 hr		19 hr		14 hr	
Cinnamic acid absorbed by tissue after 1 hr	60%		66·5%		28%		29%		72%		88%	

* Cinnamic acid (350 mc/mM) fed 4·06 × 10^6 dpm/treatment.
† CA, Chlorogenic acid; DCA, 3,5-dicaffeoylquinic acid.
‡ Sizes of leaves, stems and roots are shown in Fig. 1.
§ Half-life of chlorogenic acid in hours calculated from a semi-log of specific activity against time.

Specific activities of chlorogenic acid and 3,5-dicaffeoylquinic acid at 1, 3, 10 and 21-hr intervals after removal of the precursor are shown in Table 2. An estimation of the half-life of chlorogenic acid derived from a semi-log plot of specific activity against time is also shown. In the stem tissue and various leaf tissues analysed, the points fall on straight lines, indicating that the decay follows first order kinetics. The total amount of activity lost from chlorogenic acid during the 20-hr period of these experiments in young leaves and stems is comparable to that appearing in 3,5-dicaffeoylquinic acid (Table 3). As leaves mature, the turnover rate slows down and less of the counts disappearing from chlorogenic acid appear in 3,5-dicaffeoylquinic acid. In the root, a semi-log plot of specific activity against time does not produce a straight line; more data will be required before this can be explained.

TABLE 3. TOTAL ACTIVITY GAINED OR LOST IN VARIOUS FRACTIONS PER g DRY WEIGHT OF *Xanthium* TISSUE OVER A 20 HR PERIOD

Activity	Activity dpm × 10 ^{3} *Leaf 5	Leaf 24	Leaf 78	Leaf senescing	Stem	Root
Lost from chlorogenic acid	1832	2054	2498	4480	2110	1417
Gained by 3,5-dicaffeoylquinic acid	2252	1312	816	987	2672	607
Gained by insoluble residue	422	134	6	26	5	801

* See Fig. 1 for explanation.

As found previously in relatively mature leaves,[3] the insoluble caffeic acid fraction shows a very slow and slight labelling from cinnamic acid. In ratemeter scans of chromatograms of ether soluble components from 5 *N* NaOH hydrolysis of the alcohol-insoluble residues, no label could be seen in caffeic acid from fully mature and senescing leaves—detection limit 400 cpm. In the youngest leaf a slow increase in the labelling of this fraction to a 1000-cpm total after 21 hr could be seen, and this was slightly more pronounced in roots where approximately 2000 cpm accumulated during this time. The specific activity of the insoluble caffeic acid in all plant parts is orders of magnitude less than the combined specific activities of the soluble esters, which must mean it is not an extraction-induced artifact.

A significant proportion of the absorbed radioactivity remains in the residue after alcohol and 5 *N* NaOH extraction (Table 4). In roots and expanding leaves there is a significant rise

TABLE 4. TOTAL ACTIVITY REMAINING IN THE INSOLUBLE RESIDUE* OF *Xanthium* LEAVES, STEMS AND ROOTS AFTER ALCOHOL AND COLD 5 *N* NaOH EXTRACTION

Hours after removal of (^{14}C) cinnamic acid	Total Activity dpm × 10^{-3}/g dry wt Leaf 5	Leaf 24	Leaf 78	Leaf senescing	Stem	Root
1	499	748	171	125	471	779
3	678	822	168	140	419	1053
10	767	857	167	163	461	1120
21	921	882	177	151	476	1580

* This label is presumably in lignin.[3]

in the incorporation of this fraction over a 21 hr period. There is little increase in the labelling of similar fractions from fully expanded leaves or young stems. The most significant feature of these figures, however, is that 50 to 100 per cent of the total labelling of the fraction occurs during the first hour following application of the tracer.

$^{14}CO_2$ *Labelling Experiments*

Xanthium plants, grown as previously under continuous illumination, were defoliated to a single leaf (¾ expanded) immediately before use. Five plants were exposed to 0·5 mc of $^{14}CO_2$ (26·7 mc/mM) for 5 hr in diffuse sunlight. They were then transferred to a normal atmosphere and leaf discs taken at 0, 5, 10, 30, 60 and 180-min intervals. Soluble and insoluble esters were extracted from the lyophilized discs as described in the cinnamic acid feeding experiments.

The pattern of labelling of soluble and insoluble caffeoyl moieties from $^{14}CO_2$ was similar to that previously observed when using L-phenylalanine as precursor.[3] Caffeic acid obtained by anthocyanase (esterase) hydrolysis of the total soluble caffeoyl esters was labelled extensively. Total activity (8000 dpm/time course) remained reasonably constant during the 3 hr experiment. This is to be expected since the caffeic acid has come from both chlorogenic and 3,5-dicaffeoylquinic acid. Bound caffeic acid liberated with 5 *N* NaOH or anthocyanase was only slightly radioactive (30–60 dpm/time course) and what labelling there was changed little over the 3 hr period.

Utilization of Caffeoyl and Quinic Labelled Chlorogenic Acid

Chlorogenic acid labelled predominantly in the caffeoyl moiety (> 98 per cent) or quinic moiety (~91 per cent) was applied in water to mature leaves of otherwise defoliated *Xanthium* plants. Leaves were dipped in 0·001 per cent Tween 80 before application of the tracer, and were then held at a high humidity in continuous light. Non-adsorbed tracer was removed after 26 hrs and the leaves and other plant parts lyophilized. Dried tissue was extracted with ethanol and 5 *N* NaOH as previously.

TABLE 5. DISTRIBUTION OF RADIOACTIVITY AMONGST FRACTIONS OF *Xanthium* LEAVES FED PREDOMINANTLY QUINIC OR CAFFEOYL LABELLED CHLOROGENIC ACID FOR 26 HR

Fraction	Quinic labelled (%)	Caffeoyl labelled (%)
Alcohol soluble	3	47
5 *N* NaOH soluble*	67 (21)	17 (81)
Insoluble residue	30	36

* Percentages in parenthesis refer to the amount of counts that are transferable to ether from the acidified 5 *N* NaOH hydrolyzate.

There are major differences in the pattern of utilization of the two halves of chlorogenic acid (Table 5). Another interesting point is that 15 per cent of the total activity in the alcohol-soluble extract from the caffeoyl labelled chlorogenic acid remains in the caffeoyl moieties of chlorogenic and 3,5-dicaffeoylquinic acids. The remaining activity is in unknown compounds that separate into at least 4 bands on chromatography in *n*-butanol-acetic acid–water and *n*-butyl acetate–acetic acid–water. Chromatography of the ether soluble compounds from the 5 *N* NaOH hydrolyzate in benzene–acetic acid–water indicates that

30 to 50 per cent of the counts in this fraction are in caffeic acid in both the quinic and caffeoyl labelled chlorogenic acid feeding experiment. Alkaline nitrobenzene oxidation of the insoluble residue produces some labelled vanillin, but less than 10 per cent of the activity in the residue can be accounted for in lignin aldehydes in either experiment. An excess of cold vanillin added to the reaction mixture did not increase the yield, and recovery of the added vanillin was 50 per cent or less.

Little activity was found outside the treated leaves after the 26 hr period of treatment. No activity could be detected in the roots or stem below the inception of the petiole of the treated leaf. The short piece of stem and primordial leaves above the petiole were slightly radioactive. Total counts in the ethanol and 5 *N* NaOH solubilized fractions were 0·4 per cent of those of similar leaf fractions in the quinic labelled experiment, and 0·6 per cent in the caffeic labelled experiment.

DISCUSSION

Chlorogenic acid (3-caffeoylquinic acid) and 3,5-dicaffeoylquinic acid have previously been identified as the major phenolic acid esters in *Xanthium*.[7] This work has shown that these soluble esters are present in highest concentration in the most rapidly metabolizing tissues, i.e. in young leaves and stems. This parallels the pattern found in *Coffea*.[8] Previous studies using labelled L-phenylalanine or cinnamic acid as precursor have shown a marked turnover of chlorogenic acid in two-thirds expanded *Xanthium* leaves.[3] Much of the caffeoyl labelled chlorogenic acid was transformed to 3,5-dicaffeoylquinic acid, and some appeared to be used in the biosynthesis of lignin. Some difficulty was found, however, when making calculations using the observed half-life (14 hr) in accounting for the total amount of chlorogenic acid that should turn over during the life of the leaf.

In the present work it has been found that chlorogenic acid turns over most rapidly in young leaves and slowest, though still significantly, in fully expanded leaves. In the 11-day expansion period of a leaf from 5 to 78 cm^2, the chlorogenic acid increases from 1·08 μM to 6·14 μM/leaf, while 3,5-dicaffeoylquinic acid increases from 2·56 μM to 5·65 μM. Using an average of 3μM of chlorogenic acid with a half-life of 15 hr during this 11-day expansion period, a total of 25 μM of chlorogenic acid should turn over. During this same period 3 μM of 3,5-dicaffeoylquinic acid have been formed. If both caffeoyl moieties of the chlorogenic acid are utilized in 3,5-dicaffeoylquinic acid synthesis, this would explain the utilization of 6 μM of chlorogenic acid. This is a minimal figure since it assumes that 3,5-dicaffeoylquinic acid does not turn over. If the remaining (19 μM) were utilized in lignin synthesis, a total of approximately 1 per cent dry weight of lignin could be formed in fully expanded leaves. Looking at the distribution of label with time among the various fractions, a number of problems present themselves. The counts gained by 3,5-dicaffeoylquinic acid, presumably from chlorogenic acid, are a large percentage (33–100 per cent) of those lost from chlorogenic acid in leaves of all ages in the 1–20 hr period after removal of the tracer. Counts gained by the insoluble residue in the same tissues are only 23 per cent to less than 1 per cent of those lost from chlorogenic acid. If the previous assumptions based on the total amount of 3,5-dicaffeoylquinic acid formed from a known amount of chlorogenic acid turning over are correct, average figures of 24 per cent label in 3,5-dicaffeoylquinic acid and 76 per cent label in insoluble residues should be obtained.

[7] A. O. TAYLOR, *Plant Physiol.* **40**, 844 (1965).
[8] A. HAMIDI and H. WANNER, *Planta* **61**, 90 (1964).

Another unusual feature of the results may help explain these ambiguities. Between 60 and 95 per cent of the total labelling of the leaf insoluble residues has occurred in the 2 hr prior to the first sampling, but the rate of labelling of the residue in the succeeding 20 hr is orders of magnitude less (Table 4). These results could be explained if the site of chlorogenic acid synthesis was near or in the cell wall. When synthesized, a significant proportion of it (young > medium > old leaves) is used in lignin synthesis while the rest migrates into the cytoplasm. Once in the cytoplasm, little is available for lignin synthesis, and we see the pattern of change shown in Tables 2 and 3.

Some other evidence is in favour of this hypothesis. Stafford[9] has reported that cell wall fractions of *Phleum* will synthesize lignin from sucrose and hydrogen peroxide. Proteins occur in the primary cell wall.[10] Also, when chlorogenic acid (caffeoyl labelled) is supplied exogenously to *Xanthium* leaves, there is a very rapid incorporation of activity into an insoluble residue.[3] This rate slows down after an hour or two, and is followed by a slow, more normal, pattern of turnover, largely into 3,5-dicaffeoylquinic acid.

An attempt was made to determine whether this apparent utilization of chlorogenic acid was real, since Stafford[11] has reported that it has little promotive influence on lignin biosynthesis in first internodes of *Sorghum*. It is possible that its unusual path of entry could cause it to be oxidatively polymerized. The incorporation of label into various cell fractions from predominantly caffeoyl labelled chlorogenic acid has been compared to the incorporation of predominantly quinic labelled chlorogenic acid into similar fractions. If the binding or polymerization were non-specific, one would expect the ratio of counts in the alcohol-soluble extract compared to that in the insoluble residues to be reasonably similar wherever the chlorogenic acid were labelled. Unequal distributions could still arise however, if, once inside the cell, unequal rates of utilization of the two halves of chlorogenic acid occurred. In this work there are marked differences in the pattern of labelling (Table 5). Almost half the activity from the caffeoyl labelled chlorogenic acid has remained alcohol soluble compared to only 3 per cent of the activity from the quinic-labelled acid. This would appear to favour the idea of a normal enzymic utilization of chlorogenic acid rather than a non-specific oxidative polymerization. The fact that such a high proportion of counts from caffeoyl labelled chlorogenic acid have remained alcohol-soluble and chromatographically separable into a number of discrete compounds with behaviour unlike those of known partial oxidation products of chlorogenic acid[3] would also support this. Chlorogenic acid is so sensitive to oxidation that if reactions of this nature occurred as it penetrated the leaf, it seems likely that the whole amount used in these experiments (2 μM/leaf) would have been affected.

This evidence suggests the presence of an esterase in the cell walls and a utilization of the caffeoyl and quinic moieties in the biosynthesis of alkali soluble material and of insoluble polymers. Whether these are lignin precursors and lignin respectively must await more suitable degradation procedures. Only a small proportion of activity in the insoluble residue can be found in lignin aldehydes after nitrobenzene oxidation, but this technique is probably not very accurate when used, as here, on crude cell-wall preparations.

MacLeod and Pridham[12] have reported the movement of phenols, including caffeic acid, applied as 1 per cent w/v solutions to cut ends of main laminal veins, at virtually normal phloem translocation rates. In this work, label from both caffeoyl and quinic labelled

[9] H. A. Stafford, *Plant Physiol.* **40**, 844 (1965).
[10] D. T. A. Lamport, In *Advances in Botanical Research*, p. 204. Academic Press, New York (1965).
[11] H. A. Stafford, *Plant Physiol.* **42**, 450 (1967).
[12] N. J. MacLeod and J. B. Pridham, *Phytochem.* **5**, 777 (1966).

chlorogenic acid was found in extracts of the upper stem after 26 hr, but it accounted for only 0·4 to 0·6 per cent of the activity in similar extracts of the leaf to which the label had been applied. This reinforces an earlier tentative comment[3] that translocation is not an important factor in the metabolism of chlorogenic acid in *Xanthium* leaves. Indeed, in view of the number of labelled compounds appearing in the alcohol soluble extract after chlorogenic acid-^{14}C feeding, the small amount of translocation may not be through chlorogenic acid at all. Labelled chlorogenic and 3,5-dicaffeoylquinic acid have, however, been isolated in small amounts from stem segments in some feeding experiments.

In previous experiments with *Xanthium*[3] and potato[3, 4] using L-(U-^{14}C) phenylalanine and *trans*-(U-^{14}C) cinnamic acid, alcohol-insoluble caffeic acid esters have shown a faint and gradual increase in labelling, while soluble esters were labelled rapidly and extensively and thereafter lost activity. These observations do not suggest that the insoluble esters are precursors of the soluble esters in all plants.[2] However, "one must always be aware that the ultimate object is to discover the normal obligatory biosynthetic pathway(s) from elementary carbon sources" (Swain[13]).

Turnover experiments using $^{14}CO_2$ as precursor were carried out to learn whether insoluble caffeoyl residues would become rapidly and extensively labelled as reported in wheat and barley.[2] When $^{14}CO_2$ was used a very similar picture to that obtained when using L-phenylalanine-^{14}C was found. The caffeic acid moiety of chlorogenic acid was labelled quickly and extensively while the insoluble caffeic acid was barely labelled at all. If these insoluble esters are precursors of the soluble esters in *Xanthium*, an approximate calculation suggests that the active pool must be a very small part (< 1 per cent) of the total.

EXPERIMENTAL

Xanthium pensylvanicum (Wall.) Bonner strain, was grown in a greenhouse at 23–15° under continuous illumination consisting of natural daylight plus 400 ft-c incandescent supplementary. After germination, the seedlings were potted into 3 to 1 pumice and Sphagnum peat mixture, and supplied with an Iron EDTA modified Hoagland's nutrient solution. Leaf discs, stem sections and roots were taken from closely comparable plants 24 days after they were potted out.

After several thorough rinsings in deionized water, 1·2 cm discs punched from the leaves remained in excellent condition when kept for several days on moist filter paper. Stem sections 2 mm thick and 6 mm in diameter were cut with a pair of fixed razor blades from the internode immediately above the last fully expanded leaf (see Fig. 1). After thorough rinsing in deionized water, these sections also remained in excellent condition for several days. Clumps of secondary roots 0·3 mm in diameter were cut from the primary roots, and washed thoroughly in deionized water before use.

Trans-(U-^{14}C) cinnamic acid was prepared enzymatically from L-phenylalanine (350 mc/mM Nuclear Chicago Corp.) using a purified phenylalanine-ammonia lyase isolated from potatoes.[14] One ml of an aqueous solution of the labelled *trans*-cinnamic acid ($4{\cdot}06 \times 10^6$ dpm) was applied as 0·05 ml drops to the leaf discs and stem sections (24 per treatment), or as evenly as possible over the mass of fine roots. After 1 hr the radioactive material not absorbed by the tissue was removed from the discs or roots by rinsing 4 or 5 times in large volumes of distilled water. After being blotted dry, the discs, section or roots were placed on moist filter paper in a petri dish and maintained in the light (250 ft-c) at 20°. Samples consisting of 6 leaf discs (approx. 24 mg dry weight), 6 stem sections (approx. 33 mg dry weight) or 72 mg dry weight of roots, were taken at 1, 3, 10 and 21 hr intervals after removal of the tracer, frozen by high vacuum then thoroughly lyophilized. Isolation of chlorogenic and 3,5-dicaffeoylquinic acid from these tissues were performed as previously described.[3] Insoluble caffeic acid was liberated with 5 *N* NaOH[3] or with anthocyanase pH 3·8,[15] extracted into ether and purified by chromatography in benzene-acetic acid-water (125:72:3). Alkaline nitrobenzene oxidations were carried out using the modified micromethod of Stone and Blundell.[1]

[13] T. SWAIN, In *Biosynthetic Pathways in Higher Plants* (edited by J. B. PRIDHAM and T. SWAIN), p. 9. Academic Press, New York (1964).

[14] E. HAVIR and K. R. HANSON, *Federation Proc.* **25**, 790 (1966).

[15] J. B. HARBORNE and J. J. CORNER, *Biochem. J.* **81**, 242 (1961).

Chlorogenic acid (SA. $1{\cdot}2 \times 10^5$ dpm/μM) labelled predominantly in the caffeoyl moiety (> 98 per cent) was synthesized by feeding *Xanthium* leaves labelled cinnamic acid. *Trans*-(U-^{14}C) cinnamic acid 350 mc/mM ($4{\cdot}06 \times 10^6$ dpm) was supplied to a single mature leaf for 6 hr in the light. After lyophilization of the leaf, chlorogenic acid was extracted in ethanol and purified by paper chromatography in *n*-butyl acetate-acetic acid–water (4:1:5; ascending, in the organic phase after moistening the paper with a fine water mist) and *n*-butanol-acetic acid–water (12:3:5; descending).

Predominantly (91 per cent) quinic labelled chlorogenic acid (SA. $2{\cdot}5 \times 10^5$ dpm/μM) was synthesized in *Xanthium* leaves using $^{14}CO_2$ as precursor. Carbon dioxide was released from 0·572 mc of Ba $^{14}CO_3$ with dilute lactic acid into a 5-l. display jar containing a *Xanthium* plant with two mature leaves. After 9 hr the plant was removed, the leaves lyophilized and labelled chlorogenic acid extracted with ethanol. The chlorogenic acid was purified on a silica gel column.[16]

The concentration of the two caffeoyl conjugates was determined from the absorption of the eluates at 330 nm assuming that the millimolecular absorptivities were 20 and 40 for chlorogenic acid and 3,5-dicaffeoylquinic acid respectively.[17] Radioactivity was measured in a Packard Model 2211 scintillation counter using toluene-ethanol (7:3) containing 5 g 2,5-diphenyloxazole and 0·2 g 1,3 bis (2-[5-phenyloxazolyl])-benzene per l. Quenching was determined by channels ratio.

[16] K. R. HANSON and M. ZUCKER, *J. Biol. Chem.* **238**, 1105 (1963).
[17] J. CORSE, R. E. LUNDIN and A. C. WAISS, JR., *Phytochem.* **4**, 527 (1965).