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INCORPORATION OF $^{14}CO_2$, ^{14}C-PHENYLALANINE AND ^{14}C-CINNAMATE INTO SOLUBLE AND INSOLUBLE CINNAMIC DERIVATIVES IN *MENTHA ARVENSIS*

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Key Word Index—*Menthaarvensis*; Labiatae; mint; ethanol-insoluble cinnamic esters; caffeic acid; turnover of phenolic conjugates.

Abstract—With the successful development of methods for the isolation and purification of ethanol-insoluble cinnamic acid esters in mint it became possible to initiate kinetic, isotopic studies on purified, 'insoluble' derivatives of caffeic acid, ferulic acid and *p*-coumaric acid. Pulse-feeding experiments were conducted with $^{14}CO_2$, phenylalanine-U-^{14}C and cinnamic acid-3-^{14}C. The ferulic acid derivative exhibited a significant turnover as compared to the other insoluble derivatives which were relatively stable. Time-course tracer studies were performed to compare the turnover of soluble caffeic acid derivatives with 'insoluble' forms of caffeic acid. Caffeic acid associated with a macromolecular fraction consistently showed a higher specific activity than either soluble caffeic acid or the caffeic acid associated with a second insoluble derivative.

INTRODUCTION

BOUND, insoluble cinnamic acids were originally detected in lignin preparations[1–4] and Smith postulated that these acids occur as ester residues attached to pre-formed lignins.[1] Alkali-hydrolysable, phenolic acid conjugates were later detected in the ethanol-insoluble fraction of wheat shoots.[5] The quantitative changes in the levels of these acids during growth and development were compared with levels of 'soluble' acids occurring as hydrolysable glycosides and esters. Dynamic fluctuations in concentration were observed over an 8-week period. Subsequent tracer experiments revealed that the rate of incorporation of $^{14}CO_2$, ^{14}C-phenylalanine and tyrosine was more rapid into the 'insoluble esters' of the hydroxycinnamic acids than into the soluble esters.[6] The kinetic data indicated that 'insoluble' esters, in fact, behaved more like precursors of lignin than did the soluble esters.

Taylor and Zucker, on the other hand, have emphasized the role of the soluble esters in lignification.[7,8] Tracer studies on phenylpropanoid metabolism in *Xanthium* leaves and potato tubers indicated that chlorogenic and isochlorogenic acids were actively turning over while 'insoluble' caffeic' acid was comparatively inactive.[8] The tracers were administered to exceedingly small samples (e.g. 5 leaf discs weighing 120 mg) and the active,

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[6] EL-BASYOUNI, S. Z., NEISH, A. C. and TOWERS, G. H. N. (1964) *Phytochemistry* **3,** 627.
[7] TAYLOR, A. O. (1968) *Phytochemistry* **7,** 63.
[8] TAYLOR, A. O. and ZUCKER, M. (1966) *Plant Physiol.* **41,** 1350.

insoluble pools may not have been detected. Furthermore, the 50% aqueous methanol extract that was discarded in their fractionation procedure may have contained yet other pools of phenolic acids.

Fritig *et al.*, in studies on coumarin biosynthesis, were unable to find activity in the insoluble phenolic derivatives of tobacco tissue cultures[9] but the levels of insoluble phenolic acids were very low[10] as might be expected in tissue cultures which are not undergoing extensive lignification. On kinetic grounds, they concluded that the formation of scopoletin was via the free hydroxycinnamic acids and not through glucosides or soluble esters[9] as postulated by Brown *et al.*[11]

Fuchs *et al.* have carried out extensive studies on the response of phenolic metabolism in wheat to rust infection.[12–14] They have confirmed the previous observation that the 'insoluble' esters of wheat are more rapidly labelled than the soluble esters. When ^{14}C-phenylalanine was administered (22-hr metabolic period), the ratios of the activities in the 'insoluble' ferulic acid conjugates to the activities in the 'soluble' ferulic acid conjugates were 11:1 (susceptible, healthy leaves), 2:1 (susceptible, rust infected leaves), 9:1 (resistant, healthy leaves) and 9:1 (resistant, rust infected leaves).[12]

Using ^{14}C-phenylalanine, Ellis and Towers demonstrated that the incorporation of label into the ethanol-insoluble, phenolic esters of *Mentha* was as great as the total label in the pool of soluble rosmarinic acid.[15] The results were obtained by the following procedure: The ethanol-insoluble residue was hydrolysed in alkali, acidified, extracted with ether and the total activity in the ether phase was designated as the label in the 'ethanol-insoluble' ester pool.

On the basis of their studies, of the presence of labelled phenolic acids in ammonium sulfate precipitates and in Sephadex G200 eluates, El-Basyouni and Neish[16] and later, Hope[17] proposed that the metabolically-active, 'insoluble', phenolic acids of wheat were associated with proteins. Hope prepared buffered extracts of acetone powders of wheat and these were separated on Sephadex G25 which yielded so-called 'protein' (> 5000 g/M) and 'non-protein' (< 5000 g/M) fractions.[17] Phenylalanine-U-^{14}C was administered and samples were obtained at 1, 2 and 4 hr. During the 1–2 hr metabolic period, the specific activities of 'non-protein' *p*-coumaric and ferulic acids increased while those of 'protein' *p*-coumaric and ferulic acids decreased. However, during the 2–4 hr interval, the correlation was reversed for *p*-coumaric acid and a gradual decline was observed for both fractions of ferulic acid. Hope did not examine the specific activities of the soluble acids. Since the crude Sephadex fractions were not purified, the homogeneity of these phenolic pools is questionable and the possibility remains that the phenolic moieties were weakly associated via hydrogen bonding or adsorption. The MW limits mentioned above were established on the basis of gel chromatography in conventional buffers. Under these conditions phenolic conjugates are strongly adsorbed to the gel, true gel filtration is not operational and the estimation of MW limits can be misleading.[18]

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[18] JANSEN, J.-C. (1967) *J. Chromatog.* **28,** 12.

New methods for the isolation and purification of ethanol-insoluble cinnamic esters of mint have been described by us.[19] Accordingly, it was possible to initiate kinetic isotopic studies on purified 'insoluble' derivatives of caffeic acid (compound I), ferulic acid (compound II) and *p*-coumaric acid (compound III). Time-course tracer studies were also performed to observe the turnover, if any, of soluble caffeic acid derivatives in relation to the 'insoluble' forms of caffeic acid derived from compound I and from a high-MW fraction.

RESULTS AND DISCUSSION

Time-course Tracer Studies on Compounds I–III

Because of the batchwise procedure of isolation and the adsorptive properties of these compounds on DEAE-cellulose, determinations of absolute pool sizes was not possible. Taylor and Zucker reported that in *Xanthium pennsylvanicum* the content of chlorogenic acid, isochlorogenic acid and insoluble caffeic acid remained constant over a 24-hr period.[8] Subsequently, Taylor showed that the concentrations of these pools varied between different organs of the same plant but no significant net synthesis occurred in any of the tissues over a 21-hr period.[7] On this basis, the assumption has been made that in mint shoots there was no significant net increase of compounds I–III during the period of assimilation and metabolism of tracers.

Weighable quantities (10–30 mg) of compound I (caffeic derivative) were isolatable but the amounts of compounds II (ferulic) and III (*p*-coumaric) were too low for accurate weight determination. The relative pool sizes of compounds I–III were consistent, however, on the basis of spectrophotometric determinations of 26 samples from four, time-course, tracer studies. The estimation of the relative pool size of compounds I–III is based on the following criteria. The calculated molar extinction coefficients of caffeic, ferulic and *p*-coumaric acid differ by $\pm 10\%$ about the mean. The phenolic moieties on I–III were identified respectively as caffeic, ferulic and *p*-coumaric acid. The physical properties (adsorption, electrophoretic mobility and R_fs) of these compounds are similar and therefore, for comparisons to be made in kinetic experiments, it was assumed that the non-phenolic component was the same in each compound. Thus, the relative pool sizes were estimated by directly comparing the absorptivities of compounds I–III: using this method, the ratio of the concentrations of I:II:III was found to be 50:1:5.

Tracer experiments using $^{14}CO_2$ in light (Experiment I, Table 1) were carried out to demonstrate that compound I is labelled through 'the normal obligatory pathway(s) from elementary carbon sources'[20] and the results of Experiment I supported this hypothesis. Furthermore, after a pulse feeding, a decline in specific activity with time was obtained. Similarly, when ^{14}C-phenylalanine and ^{14}C-cinnamate were administered, compounds I–III exhibited a decline in specific activity with time which pointed to a turnover of these compounds (Experiments II and III).

Compound II, the ferulic acid-containing conjugate, consistently possessed the highest specific activity in a given experiment. This is interesting because it is presumably more closely related to a lignin precursor than either I or III.

The significant difference between the specific activities of the various compounds is especially noticeable in Experiment III. The relatively small pool of compound II may account for its high specific activity. This would not explain, however, the fact that the

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specific activity of compound III is approximately the same as that of compound I although it has a pool size one-tenth as great.

A further experiment was carried out with ^{14}C-phenylalanine to discover whether a turnover of compounds I–III was occurring. Figure 1 shows that the ferulic acid-containing conjugate (compound II) exhibited a rapid increase in specific activity followed by a decline. This would seem to indicate a significant turnover as compared to compounds I and III which are stable and of much lower specific activity.

TABLE 1. THE SPECIFIC ACTIVITIES OF INSOLUBLE CINNAMIC DERIVATIVES AT VARIOUS TIME INTERVALS AFTER THE ADMINISTRATION OF $^{14}CO_2$, PHENYLALANINE-U-^{14}C OR CINNAMIC ACID-3-^{14}C

		Specific activity*					
		Compound I (caffeic acid)		Compound II (ferulic acid)		Compound III (*p*-coumaric acid)	
Experiment	Tracer	6 hr	12 hr	6 hr	12 hr	6 hr	12 hr
I	$^{14}CO_2$†	9·7	6·4	—	—	—	—
II	^{14}C-Phenylalanine‡	6·6	5·0	14·8	13·1	9·7	8·0
III	^{14}C-Cinnamic acid§	10·2	6·8	64·3	25·1	5·0	3·3

* Specific activity expressed as dpm/Absorbance unit. Absorptivity calculated for 200 ml aqueous solutions. Compound I, 1·00 mg/200 ml, Absorbance = 0·10.

† 4·9 mCi/mM, 37·5 μCi per sample.

‡ 375 mCi/mM, 25 μCi per sample.

§ 9·0 mCi/mM, 25 μCi per sample.

Time-course Tracer Studies on Various Pools of Caffeic Acid

Kinetic isotopic experiments were performed to compare the activity of two 'insoluble' forms of caffeic acid with soluble caffeic acid. The 'insoluble' pools of caffeic acid were derived from compound I and from the high molecular weight (HMW) fraction[19] by alkaline hydrolysis. Soluble caffeic acid in *Mentha* occurs mainly in the form of rosmarinic acid which is found in the acetone-soluble pool and this fraction was also subjected to alkaline hydrolysis. Ellis and Towers reported that rosmarinic acid could be isolated in 0·2% (fr. wt) yield from *M. arvensis*.[15] The relative size of the various pools of caffeic acid were estimated to be 10 (soluble caffeic acid):1 (caffeic acid from compound I):1 (caffeic acid from the HMW fraction) on the basis of spectroscopic determinations of caffeic acid obtained after alkaline hydrolyses. The concentrations of the caffeic acid associated with 'the cyanidin glucoside'[19] were too low for accurate determinations.

Table 2 describes the various isotopic treatments in Experiments V–VII (Fig. 2) and a comparison is made between the specific activities of the various pools of caffeic acid after pulse-feeding for 1·5 hr followed by a metabolic period of 10·5 hr. The specific activities of the soluble pool of caffeic acid are of the same order of magnitude as those obtained for the caffeyl moiety of rosmarinic acid in comparable experiments with mint[15] and the results were reproducible. For example, in Experiment IV (see Fig. 1) the incorporation of ^{14}C-phenylalanine into the caffeyl moiety of compound I was found to be 0·36, 0·70, 0·76 and 0·50 μCi/mM at 3, 6, 12 and 24 respectively. In the same experiment the values for soluble caffeic acid were 1·62, 2·08, 3·51 and 1·95 μCi/mM respectively. The

results indicate that cinnamate-^{14}C is a better precursor than either $^{14}CO_2$ or phenylalanine-^{14}C for caffeic acid.

Extremely high dilution values were obtained with phenylalanine-^{14}C. This can be attributed in part to the large amounts of plant material (50 g) used in each treatment. The high specific activity of the precursor, the low dosage and the large pool sizes of products undoubtedly contribute to the high dilution values.

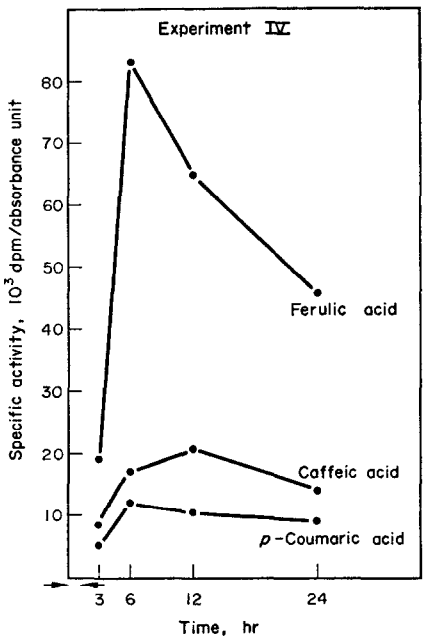

FIG. 1. CHANGES WITH TIME IN THE SPECIFIC ACTIVITIES OF ETHANOL-INSOLUBLE (CAFFEIC ACID) COMPOUNDS I, II (FERULIC ACID) and III (*p*-COUMARIC ACID) AFTER THE ADMINISTRATION OF 12·5 cm^3 OF L-PHENYLALANINE-U-^{14}C TO EACH OF 50-g LOTS OF *Mentha arvensis* SHOOTS (EXPERIMENT IV).

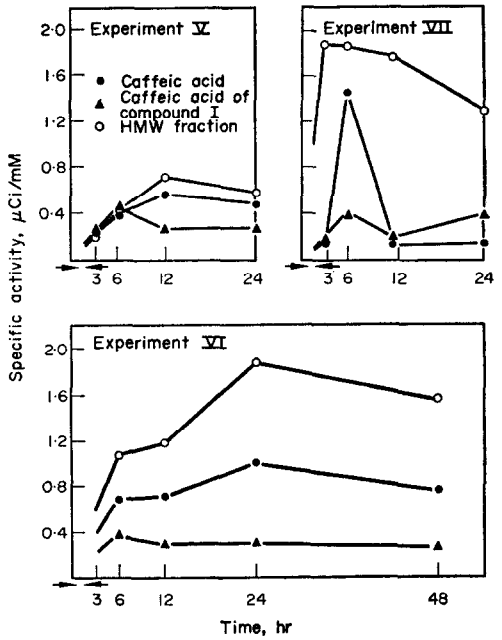

FIG. 2. CHANGES WITH TIME IN THE SPECIFIC ACTIVITIES OF SOLUBLE CAFFEIC ACID (●—●—●), THE CAFFEIC ACID OF COMPOUND I (▲—▲—▲) AND THE CAFFEIC ACID OF THE HMW FRACTION ○—○—○) AFTER THE ADMINISTRATION OF $^{14}CO_2$ (EXPERIMENT V), PHENYLALANINE-U-^{14}C (EXPERIMENT VI) AND CINNAMATE-3-^{14}C (EXPERIMENT VII).

Experiment V. The dramatic decline in specific activity of 'insoluble' ferulic and *p*-coumaric acids found in wheat was not observed with the 'insoluble' forms of caffeic acid in mint. The general picture with respect to the soluble caffeic acid and the caffeic acid of the HMW fraction is one of gradual labelling from $^{14}CO_2$ followed by a relative stability in the amount of label in these pools. On the other hand, the specific activity of the caffeyl moiety of compound I is lower at the 12- and 24-hr-intervals.

Experiment VI. The results with ^{14}C-phenylalanine were similar to those with $^{14}CO_2$. Although the specific activity of the caffeic acid from the HMW fraction was twice as great as the specific activity of soluble caffeic acid at the 24-hr interval, one must bear in mind that the pool size of the latter is approximately 10 times as great. The caffeic acid of compound I exhibited little if any turnover and in this respect, resembles the behaviour

of the 'insoluble' caffeic acid of *Xanthium pennsylvanicum* as reported by Taylor and Zucker.[8] The caffeic acid of the 'insoluble' HMW fraction, however, consistently exhibited a higher specific activity than the soluble caffeic acid.

Experiment VII. The oustanding feature of Experiment VII (feeding cinnamate-3-^{14}C) was the turnover pattern of the caffeic acid derived from the HMW fraction. Thus a rapid increase was followed by a decline in specific activity. The rise and fall is comparable to the behaviour of chlorogenic acid in *Xanthium* reported by Taylor and Zucker after pulse-feeding cinnamic acid-^{3}H.[8]

The *p*-coumaryl and ferulyl moieties of the analogous 'insoluble' and soluble pools were not examined kinetically because of the extremely low concentrations of these conjugates. Experiments I–IV pointed, however, to an active turnover of compound II.

TABLE 2. INCORPORATION* OF RADIOACTIVE COMPOUNDS INTO CAFFEIC ACID OBTAINED FROM VARIOUS POOLS IN *Mentha arvensis*

				Caffeic acid					
				Soluble		Compound I		HMW fraction	
Experiment No.	Compound fed	Specific activity (mCi/mM)	Activity fed (μCi)	Specific activity (μCi/mM)	Dilution	Specific activity (μCi/mM)	Dilution	Specific activity (μCi/mM)	Dilution
V	$NaH^{14}CO_3$	4·9	150	0·57	$8·6 \times 10^3$	0·26	$1·9 \times 10^4$	0·71	$6·9 \times 10^3$
VI	L-Phenylalanine-U-^{14}C	375	25†	0·71	$5·3 \times 10^5$	0·28	$1·4 \times 10^6$	1·17	$3·2 \times 10^5$
VII	Cinnamic acid-3-^{14}C	9·0	25†	0·13	$7·1 \times 10^4$	0·29	$3·1 \times 10^4$	1·78	$5·1 \times 10^3$

* After pulse-feeding for 1·5 hr followed by a metabolic period of 10·5 hr.
† Administered to each 50-g lot of *Mentha arvensis*.

Are these compounds concerned with lignin biosynthesis and is compound II an 'active, insoluble ester' directly involved in the transfer of phenylpropanoid units to lignin? If these compounds are involved in lignin biosynthesis how can this be reconciled with evidence indicating that cinnamyl alcohols such as coniferyl alcohol can be peroxidatively incorporated into model lignins?[21,22] Experiments with potato parenchyma have demonstrated lignin formation *in vitro* from precursors such as ferulic and *p*-coumaric acids.[23] Nozu has emphasized that the essential requirements for a lignin precursor are; (a) a free phenolic hydroxyl group, (b) a vinyl linkage in the side chain of the phenolic moiety, and (c) a free 5 position in the benzene nucleus.[24] In other words, the alcoholic function in the side chain apparently is not essential and it is conceivable that esterified forms of phenolic acids could participate in lignin formation.

We wish to emphasize, however, that there is not much sense in considering whether or not 'insoluble' pools of cinnamic acids are lignin precursors, as has been done in earlier work until the various pools are isolated and analysed separately for activity. For example, in mint two insoluble caffeyl pools consistently exhibited different specific activities. If other plants are like mint, and we think that they might be, the task of doing kinetic work on the various pools of phenolic acids appears formidable.

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EXPERIMENTAL

Plant material. Mentha arvensis L. was grown under greenhouse conditions supplemented with a 16 hr photoperiod. Non-flowering shoots, 4–6 internodes in length were harvested. Freshly cut shoots were recut under H_2O before use.

Radioactive compounds and their administration. L-Phenylalanine-U-^{14}C and $NaH^{14}CO_3$ were purchased from New England Nuclear, Boston, Mass. Cinnamic acid-3-^{14}C was purchased from Schwartz Bioresearch, Orangeburg, New York. Solid isotopes were administered over 1·5 hr in 3 ml 0·02 M pH 7 phosphate buffer under constant illumination (9000 lx) and temperature (19°) to 50-g lots of mint shoots and washed with H_2O. Tracer studies with $^{14}CO_2$ were carried out in a closed chamber (Jencons No. 4 desiccator); 150 μCi $^{14}CO_2$ being given for 1·5 hr to four lots of cut shoots of *Mentha* held in beakers with their cut ends in H_2O. The shoots were then allowed to metabolize under atmospheric conditions.

Isolation of compounds I–III, soluble caffeic acid and the caffeic acid of the HMW fraction. Methods for the isolation and purification of compounds I–III from acetone powders of mint have been described.[19] Caffeic acid was obtained by the following procedures: The acetone extract was concentrated to dryness, suspended in boiling H_2O, filtered hot through a bed of Celite and the filtrate freeze-dried. The freeze-dried powder was hydrolysed for 6 hr at room temp. in 2 N NaOH under N_2, acidified and extracted continuously with Et_2O for 12 hr. The Et_2O extract was dried *in vacuo*, redissolved and in EtOH developed by 2-D PC in C_6H_6–HOAc–H_2O (10:7:3) and 2% HCOOH. This was followed by extended chromatography in the former solvent. The HMW fraction was isolated from the second acetone powder[19] by extraction with phenol–HOAc–H_2O (1:1:1, 150 ml) filtration through Miracloth and the filtrate was dialysed against 0·03% mercaptoethanol in 0·03% HOAc for 60 hr with 3 × 4 l. changes of acid and the bag contents were freeze-dried. The bag contents were hydrolysed as above and the caffeic acid isolated.

Specific activity determinations. The concentrations of all compounds were determined spectrophotometrically[19] in H_2O or EtOH, an aliquot of the same solution being used for liquid scintillation counting. Caffeic acid was obtained from compound I by alkaline hydrolysis with excess $NaBH_4$.[19] The specific activity of a sample of caffeic acid from each experiment was found to be constant during three successive crystallizations from H_2O.

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