Biochem. J. (1970) **118**, 291–297
Printed in Great Britain

Biogenesis of Rosmarinic Acid in *Mentha*

By B. E. ELLIS* and G. H. N. TOWERS
Department of Botany, University of British Columbia, Vancouver 8, B.C., Canada

(*Received* 23 *February* 1970)

The biogenesis of rosmarinic acid (α-*O*-caffeoyl-3,4-dihydroxyphenyl-lactic acid), the second most common ester of caffeic acid in the plant kingdom, was studied in *Mentha arvense* and *Mentha piperita*. Administration of ^{14}C-labelled compounds showed that, whereas the caffeoyl moiety was formed from phenylalanine via cinnamic acid and *p*-coumaric acid, the 3,4-dihydroxyphenyl-lactic acid moiety was formed from tyrosine and 3,4-dihydroxyphenylalanine. Time-course studies and the use of labelled rosmarinic acid showed that endogenous rosmarinic acid had a low turnover rate. The caffeoyl moiety did not appear to contribute to the formation of insoluble polymers, as has been suggested for chlorogenic acid in other plants.

The hydroxylated and methoxylated derivatives of cinnamic acid form the most important and most studied pool of phenolic intermediates in plant tissues, since they give rise to the coumarins, flavonoids, lignins, benzoic acid derivatives and other phenols. Plants synthesize these aromatic compounds almost exclusively from the amino acids phenylalanine and tyrosine, especially the former (Neish, 1961). The ability to deaminate phenylalanine to cinnamic acid appears to be universal in higher plants, but the conversion of tyrosine into *p*-coumaric (4-hydroxycinnamic) acid is generally low outside of the Gramineae and cannot be demonstrated in many groups of plants (Rosa, 1966).

Tyrosine is hydroxylated in some plants to form DOPA† (Kovacs & Jindra, 1964). A few plants have yielded acetone-dried powders that will deaminate DOPA to caffeic acid (3,4-dihydroxycinnamic acid) (MacLeod & Pridham, 1963; Rosa, 1966), but whether this reaction is of any significance *in vivo* is unknown. The reported distribution of DOPA in the plant kingdom is limited (Karrer, 1958) and it is known only as a precursor to some alkaloids (Leete, 1966) and to the betacyanin pigments (Horhammer, Wagner & Fritzsche, 1964).

Phenyl-lactic acid and phenylpyruvic acid are metabolically active in plants and have been demonstrated to give rise to lignins (Wright, Brown & Neish, 1958). There has been no report, however, of a direct dehydration of phenyl-lactic acid to cinnamic acid with an enzyme system from plants. With the discovery of phenylalanine ammonia-lyase (Koukol & Conn, 1963) it has been assumed generally that phenylalanine, phenyl-lactic acid and phenylpyruvic acid form a readily interchanging pool (Gamborg & Wetter, 1963) from which phenylalanine provides the main, or sole, entrance to the cinnamic acids. The same situation prevails with respect to tyrosine, *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyl-lactic acid.

In plants lacking appreciable tyrosine ammonia-lyase activity, the distribution of label from radioactive phenylalanine as compared with tyrosine is strikingly different within the non-nitrogenous metabolites. Label from phenylalanine is normally incorporated predominantly into the phenolic compounds, whereas very little label from tyrosine appears in these compounds (McCalla & Neish, 1959*a*,*b*). The label from tyrosine is usually spread throughout the organic acids and carbohydrates, indicating extensive degradation and possibly aromatic-ring cleavage (Ibrahim, Lawson & Towers, 1961). Although an enzyme system isolated from spinach will hydroxylate phenylalanine to form tyrosine (Nair & Vining, 1965), there has been no indication from tracer studies *in vivo* that this takes place to any extent in the intact plant (McCalla & Neish, 1959*a*,*b*). The fates of these two amino acids are, then, largely distinct, except when tyrosine or DOPA ammonia-lyase activity is present.

The cinnamic acids ordinarily do not occur free in higher-plant tissues in more than trace quantities. Instead, they are found as esters (Harborne & Corner, 1961; El-Basyouni & Neish, 1966) or glycosides (Runeckles & Woolrich, 1963).

* Present address: Institut für Pflanzenphysiologie, Rühr-Universitat, Bochum, West Germany.

† Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; DOPL, 3,4-dihydroxyphenyl-lactic acid.

Caffeic acid, one of the very widely accumulated hydroxycinnamic acids in plants, occurs most commonly as esters of quinic acid (Sondheimer, 1958). It has also been found esterified with shikimic acid (Levy & Zucker, 1960; Fritig, 1968), tartaric acid (Scarpati & Oriente, 1958*a*; Ribereau-Gayon, 1965), malic acid (Scarpati & Oriente, 1960), glucose (Harborne & Corner, 1961), glucosamine (Bergmann, Thies & Erdelsky, 1965), and 'ethanol-insoluble' compounds (El-Basyouni, Neish & Towers, 1964). The ester of DOPL, namely rosmarinic acid (Scheme 1), is found within a limited number of plant families, notably the Lamiaceae and Boraginaceae (Harborne, 1966; Hiller & Kothe, 1967), and is the second most common caffeoyl ester reported to occur in plants. It was originally isolated from *Rosmarinus officinalis* (Scarpati & Oriente, 1958*b*).

Caffeic acid is an established intermediate in the pathway leading from phenylalanine to coniferyl alcohol and sinapyl alcohol, compounds that appear to be the immediate precursors of the lignins, but the extent to which the pools of esterified caffeic acid participate in this pathway is not clear. In *Salvia*, tracer studies suggested that only a small fraction of the total caffeic acid was turning over rapidly, and that this represented possibly free caffeic acid as opposed to the large pool of the esterified compound (McCalla & Neish, 1959*b*).

In wheat shoots, on the other hand, studies with $^{14}CO_2$ incorporated during photosynthesis and with administered [^{14}C]phenylalanine showed that a pool of 'ethanol-insoluble' cinnamoyl esters became labelled much more rapidly than the soluble esters. The ethanol-insoluble cinnamoyl esters also lost label more rapidly when the radioactive carbon source was removed. It was suggested that these insoluble esters could be precursors to the lignins (El-Basyouni *et al.* 1964; El-Basyouni & Neish, 1966). Similar results were obtained by Rohringer & Samborski (1967) studying the effects on phenolic metabolism of wheat plants exposed to rust infection. Susceptible-reacting plants, however, differed from healthy wheat shoots in showing more activity in the soluble esters than the insoluble pool when fed with labelled phenylalanine.

Work on the biosynthesis of scopoletin in tobacco tissue cultures has demonstrated that the trace quantities of free cinnamic acids are the metabolically most active forms, the soluble esters accumulating label more slowly and the insoluble esters showing little labelling from either phenylalanine or cinnamic acid (Fritig, 1968).

In *Xanthium*, the caffeoyl esters do not appear to be metabolic end products but turn over, with a half-life for 3-*O*-caffeoylquinic acid of about 14h (Taylor & Zucker, 1966; Taylor, 1968). This ester is possibly a precursor to 3,4-di-*O*-caffeoylquinic acid, and it was suggested that perhaps both compounds were substrates for polymerization reactions that lead to the production of lignins. The 'alcohol-insoluble' esters contained comparatively little label from phenylpropanoid precursors, but the use of aqueous methanol in the extraction procedure (Taylor & Zucker, 1966) instead of 80% ethanol makes it difficult to compare this result with the earlier work on wheat.

If caffeic acid esters really play an active metabolic role in plants it becomes desirable to examine the other less well-known esters. Rosmarinic acid is particularly interesting in view of its accumulation in certain plants and because nothing is known about the source of the DOPL moiety.

MATERIALS AND METHODS

Plant material. Rosmarinus officinalis L. was grown on the campus of the University of British Columbia, Vancouver 8, B.C., Canada. Mint (*Mentha arvense* L.) was normally grown in flats in the departmental greenhouses under daylight supplemented by 16h-day fluorescent lighting. *Mentha piperita* L. and *M. arvense* that were used in feeding experiments with labelled rosmarinic acid were growing outdoors in June and July. Shoots were chosen that carried five to seven pairs of fully expanded leaves but no flower heads. The freshly cut shoots had their ends recut under water before use.

Labelled compounds and their administration. L-[U-^{14}C]-Phenylalanine, DL-[2-^{14}C]phenylalanine, L-[G-^{3}H]phenylalanine and L[G-^{3}H]tyrosine were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [2-^{14}C]-Cinnamic acid was purchased from International Chemical and Nuclear Corp., Irvine, Calif., U.S.A. DL-[3-^{14}C]-Tyrosine, DL-[2-^{14}C]DOPA, [2-^{14}C]malonic acid and $Ba^{14}CO_3$ were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. The radiochemical purity of the ^{14}C-labelled amino acids was checked by paper chromatography and strip-scanning. *p*-[2-^{14}C]Coumaric acid and [2-^{14}C]caffeic acid were prepared by the condensation of the appropriate benzaldehyde with [2-^{14}C]-malonic acid in pyridine with a trace of piperidine (Austin & Meyers, 1965). The products were purified by crystallization from water, and for caffeic acid by t.l.c. and lead acetate precipitation as well.

The acids were administered as their ammonium salts in aqueous solution (0.5–1.0ml) to 5–10g of mint shoots under constant illumination (9000 lx) at 22°C. The solution was normally completely taken up in ½–2h and was followed with water.

Isolation of rosmarinic acid and caffeic acid. Rosmarinic acid was isolated on a large scale by using the lead acetate precipitation technique employed in its original isolation (Scarpati & Oriente, 1958*b*).

The ester could be isolated from 5–10g of mint shoots by use of the above technique on a much smaller scale. After the appropriate incubation period with a labelled precursor, the shoots were homogenized in boiling 95% (v/v) ethanol and extracted until colourless. The ethanolic extract was evaporated to dryness, suspended in a small volume of hot water (15ml) and filtered through Celite.

The filter cake was washed with hot water and the combined filtrates were treated with aq. 20% (w/v) neutral lead acetate until no further precipitate appeared. The precipitate was separated by centrifugation and washed twice with water by resuspension and centrifugation. The resuspended precipitate was then decomposed by bubbling H_2S through the suspension. Decomposition was completed by heating on a steam bath and the precipitate of PbS was removed by filtration while still hot. The resulting solution of 'catechols' or polyphenols was extracted three times with a small volume of ether. The ether extract was evaporated to dryness, taken up in a minimal volume of hot water and filtered through decolorizing charcoal on a small sintered-glass funnel. The filtrate was stored at 4°C and crystallization of the rosmarinic acid initiated by seeding with a minute particle of the pure compound. The ester was recrystallized from water and was chromatographically pure after one recrystallization. The yield of purified rosmarinic acid from one batch of shoots varied from 2 to 10mg.

The distribution of label between the two moieties of the ester was determined by hydrolysis and chromatographic isolation of the caffeic acid by t.l.c. on Avicel plates. Most hydrolyses were carried out in 2M-NaOH under N_2 for 2h. Later it was found that pectinase (Levy & Zucker, 1960) hydrolysed the ester overnight with better yields of products. The hydrolysis products were extracted into ether after acidification and the extract chromatographed on Avicel plates with chloroform shaken with 0.2 vol. of water–methanol–formic acid (21:125:4, by vol.) as solvent. Multiple development separated caffeic acid from residual rosmarinic acid, DOPL and esculetin [the coumarin formed by light-activated cyclization of *cis*-caffeic acid (Kagan, 1966)]. The caffeic acid band, easily detected by its bright-blue fluorescence under 366nm u.v. light, was eluted with 95% (v/v) ethanol.

Specific-radioactivity determination. The concentration of rosmarinic acid or caffeic acid in 95% (v/v) ethanol solution was determined spectrophotometrically at 331nm and 325nm respectively. The radioactivity of a portion of the same solution was determined by liquid-scintillation counting. Blanks for compounds isolated by t.l.c. were prepared by elution of bands at the same R_F on blank plates developed in the appropriate solvent.

RESULTS AND DISCUSSION

Isolation of rosmarinic acid. Attempts to obtain a sample of pure rosmarinic acid from *Mentha* by preparative paper chromatography were unsuccessful because of oxidation of the compound during manipulation. The ester appeared to be immobile on polyamide and charcoal columns with the usual solvents.

By using the lead acetate precipitation technique applied in the original isolation of rosmarinic acid from *R. officinalis* a white crystalline solid was obtained in 0.1% yield (fresh wt.) from *R. officinalis* (reported 0.01–0.02%) (Scarpati & Oriente, 1958*b*) and in 0.2% yield from *M. arvense*. The isolated products were compared with samples of authentic rosmarinic acid (courtesy of Dr M. L. Scarpati and Dr K. Hiller) and were shown to have the same melting point, u.v. and i.r. spectra and chromatographic behaviour. The melting point of 204°C reported by Scarpati & Oriente (1958*b*) must be incorrect since the sample supplied by M. L. Scarpati, a sample supplied by K. Hiller and both of our preparations all melted at 172–174°C.

Hydrolysis of the isolated ester with sodium hydroxide or pectinase yielded caffeic acid (identified by m.p., u.v. spectrum and chromatographic behaviour) and an uncrystallizable compound with $\lambda_{max.}$ 284nm (in ethanol) that gave the colour reactions of an unconjugated catechol nucleus. The compound, DOPL, is very water-soluble and unstable to high pH. A reference sample for chromatographic purposes was isolated from hydrolysed rosmarinic acid by column chromatography (Avicel, 2% formic acid) of an ether-extracted hydrolysate.

Biosynthetic studies. Whereas on the basis of current knowledge of cinnamic acid biochemistry the caffeic acid moiety was expected to arise from phenylalanine via cinnamic acid, the possible routes to the formation of DOPL were more numerous.

In a preliminary experiment, both labelled phenylalanine and labelled tyrosine fed to *M. arvense* for 24h were incorporated into rosmarinic acid. Radioautography of the hydrolysis products after chromatography indicated that phenylalanine was incorporated into the caffeoyl moiety whereas tyrosine was converted into DOPL. This

Table 1. *Incorporation of phenylpropanoid compounds into rosmarinic acid in* M. arvense *L.*

Radioactive compounds were administered to shoots for 8h under constant illumination (9000lx) at 22°C.

Precursor fed	Radioactivity fed (μCi)	Rosmarinic acid sp. radioactivity (μCi/mmol)	Dilution	% of label in caffeic moiety
[2-^{14}C]Phenylalanine (2.5mCi/mmol)	2	1.88	1200	96.8
[3-^{14}C]Tyrosine (6.7mCi/mmol)	2	3.67	1850	3.7
[2-^{14}C]Cinnamic acid (1.15mCi/mmol)	2	3.00	407	102
4-Hydroxy[2-^{14}C]cinnamic acid (65.8μCi/mmol)	1	0.62	126	99
[2-^{14}C]Caffeic acid (121μCi/mmol)	1	0.51	236	98
[2-^{14}C]DOPA (4.07mCi/mmol)	2	3.32	1280	2

was confirmed in subsequent feedings for 8h (Table 1). The problems inherent in accurately measuring caffeic acid concentrations spectrophotometrically [the readily interconvertible *cis*- and *trans*-isomers have different ϵ values (Kahnt, 1966)] make it difficult to decide if the slight interconversion of phenylalanine and tyrosine implied by the label distribution is real.

[^{14}C]Cinnamic acid and *p*-[^{14}C]coumaric acid were incorporated more efficiently than phenylalanine and labelled only the caffeoyl moiety. On the other hand, [^{14}C]DOPA proved to be as good a precursor to rosmarinic acid as tyrosine (Table 1), indicating that the pathway did not directly involve 4-hydroxyphenyl-lactic acid or 4-hydroxyphenylpyruvic acid.

Direct esterification of caffeic acid with aliphatic alcohols is a known mode of caffeic ester formation (Harborne & Corner, 1961). Studies on chlorogenic acid biosynthesis have suggested that an alternative route to these esters is via the *p*-coumarylquinic acid ester (Levy & Zucker, 1960), and this may be the major route in some cases (Runeckles, 1963; Steck, 1968; Kojina, Minamikawa, Hyodo & Uritani, 1969). With DOPA as a probable intermediate in the formation of DOPL, the involvement of *p*-coumaroyl-DOPL in the synthesis of rosmarinic acid is a possibility. Earlier esters are unlikely since ring hydroxylation of the cinnamoyl moiety evidently precedes esterification as indicated by the good incorporation of *p*-[^{14}C]coumaric acid.

Careful examination of the phenolic compounds extracted from *Mentha* failed to show any sign of such an ester, which would be expected to show the typical fluorescence of *p*-coumaric acid (blue when viewed under 366nm-u.v. light with ammonia) and a strong colour reaction between the DOPL hydroxyl groups and diazotized *p*-nitroaniline (Bray, Thorpe & White, 1950). When ^{14}C-labelled shikimic acid, phenylalanine or tyrosine were fed to *Mentha* for 3h and the *o*-dihydroxy compounds were isolated and chromatographed followed by radioautography, rosmarinic acid was labelled from each of the three precursors and no other phenolic compounds contained appreciable label. The feeding time may have been sufficiently long, however, to allow the label to pass through a small pool of *p*-coumaroyl ester.

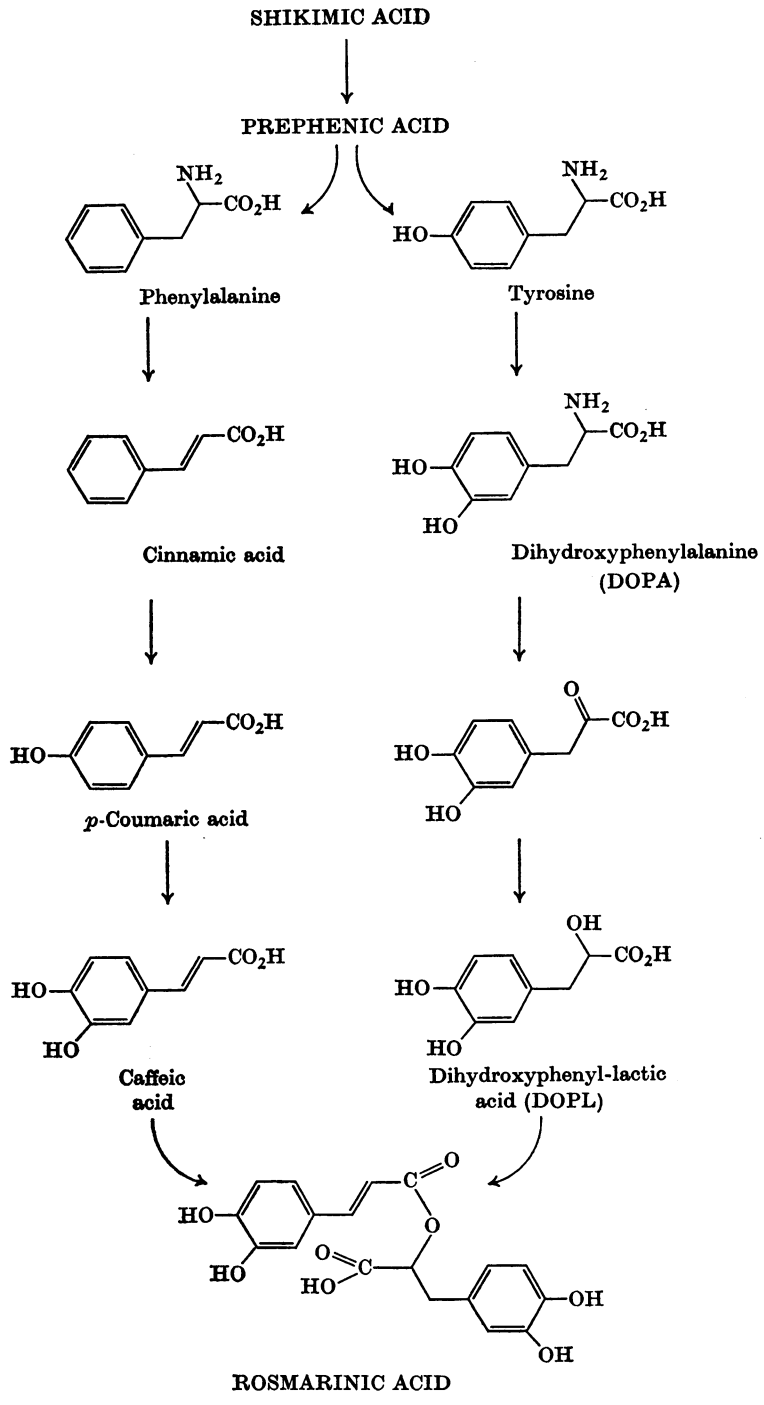
One of the main criteria used in studies on chlorogenic acid formation for implicating the intermediate nature of a *p*-coumaroyl ester has been the relatively low incorporation of administered caffeic acid. The results have not been consistent, however, and have varied from caffeic acid being incorporated with less dilution than *p*-coumaric acid (Gamborg, 1967) to its being incorporated with much greater dilution and possibly even being degraded and resynthesized (Runeckles, 1963). The picture is complicated by the frequent destruction of part of the administered caffeic acid by polyphenol oxidases as it passes through cell membranes or when it comes into contact with injured tissue, such as leaf discs, tuber discs or cut shoots.

[^{14}C]Caffeic acid administered to *Mentha* was incorporated specifically into the caffeoyl moiety of rosmarinic acid with an average dilution of about 240 times (four replicates), about twice that of *p*-coumaric acid. Considering the losses encountered in feeding such a labile compound, however, direct esterification of caffeic acid with DOPL is probably the main route of biosynthesis of rosmarinic acid in *Mentha* (Scheme 1).

Time-course studies. In a closed illuminated system, 50 shoots of *M. arvense* were exposed for 2h to continuously circulating air containing 150μCi of $^{14}CO_2$ (released from $Ba^{14}CO_3$ by addition of dilute acid). The specific radioactivities of the ester and of its caffeoyl moiety were checked over 40h. Fig. 1 shows the results from two experiments. In Expt. 1 the label reached the rosmarinic acid more slowly than in Expt. 2, which may reflect differences in age or physiological condition of the plants at that time. The short decline in specific radioactivity levelled off and little change was seen over 40h. The initial peak may have been produced by the pulse of label passing through the pools of free precursors such as monosaccharides and aromatic amino acids. The label initially incorporated into other compounds would reach these precursors more slowly through metabolic turnover and help to maintain the concentration of radioactivity in the ester. The uptake of $^{14}CO_2$ by the 40–50 shoots in the chamber was undoubtedly not completely uniform and some specific-radioactivity variation from this is unavoidable. The general picture, then, is one of rapid labelling from $^{14}CO_2$ followed by relative stability of the amount of label in this pool.

The distribution of label between caffeic acid and DOPL varied from about 70% in the caffeoyl moiety in the early part of the experiments to 52–60% in the caffeoyl moiety after 40h, indicating that the cinnamoyl compounds become labelled somewhat faster than the DOPL–DOPA pool. The relatively large dilution of DOPA as a labelled precursor (Table 1) suggests that the limiting step may be transamination or reduction.

With [U-^{14}C]phenylalanine as a precursor, there is a definite indication of turnover in the rosmarinic acid pool (Fig. 2*a*), but it is still not very rapid. The incorporation of label from phenylalanine into the 'ethanol-insoluble' ester pool in *Mentha* was demonstrated (Fig. 2*b*) with at least as much label in this pool as in the estimated total pool of rosmarinic acid over 2h. The relationship between these pools was not examined further.



Scheme 1. Most probable biosynthetic route from phenylalanine and tyrosine to rosmarinic acid.

Finally, in an attempt to test whether rosmarinic acid was being used as a substrate for the formation of insoluble polymers, e.g. lignin, labelled rosmarinic acid was prepared biosynthetically. The comparatively low specific radioactivities obtained from ^{14}C-labelled precursors made it necessary to resort to ^{3}H-labelling.

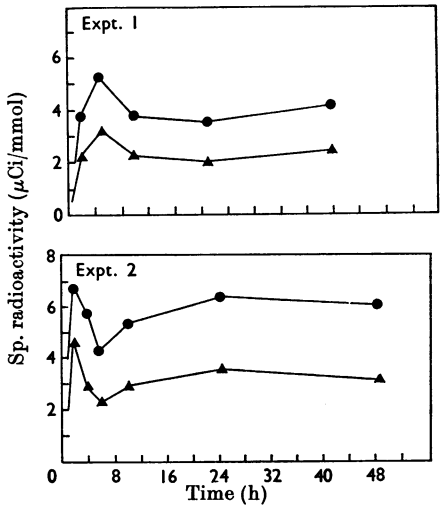


Fig. 1. Changes in the specific radioactivity of rosmarinic acid in *Mentha* after photosynthesis in $^{14}CO_2$. $^{14}CO_2$ (150μCi) was administered to 50 shoots in light (9000lx) for 2h. Samples were harvested immediately after this and again after various periods of photosynthesis in the absence of $^{14}CO_2$. ●, Sp. radioactivity of rosmarinic acid; ▲, sp. radioactivity of the caffeoyl moiety of rosmarinic acid.

[G-^{3}H]Phenylalanine (250μCi; 0.2mg) and [G-^{3}H]tyrosine (250μCi; 0.2mg) were administered to separate samples of *M. piperita* for 2h and the rosmarinic acid was isolated and purified. In each case the rosmarinic acid was specifically labelled in one moiety or the other. Each sample was readministered to *Mentha* for two feeding periods: (i) the length of time required to take up the solution of rosmarinic acid, and (ii) 24h later (Table 2).

The label from rosmarinic acid (caffeoyl moiety, ^{3}H-labelled) remained entirely in the ethanol-soluble compounds over 24h. The specific radioactivity of the isolated rosmarinic acid showed a small decrease with time, suggesting some turnover in the caffeoyl moiety. The label from rosmarinic acid (DOPL moiety, ^{3}H-labelled) in the soluble compounds, however, showed a marked decrease over 24h, as did the specific radioactivity of the isolated ester. An attempt was made to locate labelling of insoluble fractions but with little success. An increase in the labelling of the 'insoluble ester' pool (El-Basyouni, Neish & Towers, 1964) was evident but was apparently too small to account for the decrease noted above. No trace of label could be detected in the Klason lignin (or soluble hydrolysate) prepared from the 24h rosmarinic acid (DOPL moiety, ^{3}H-labelled) sample. Since the shoots used were young actively growing material containing lignin (approx. 1% of fresh wt.) it is reasonable to assume that lignin was actually being synthesized during the feeding period. It is possible that the lability of DOPL itself, or of its tritiated protons, at high pH produces large losses during procedures such as the hydrolysis of 'insoluble esters' and thus yields low results. There is, how-

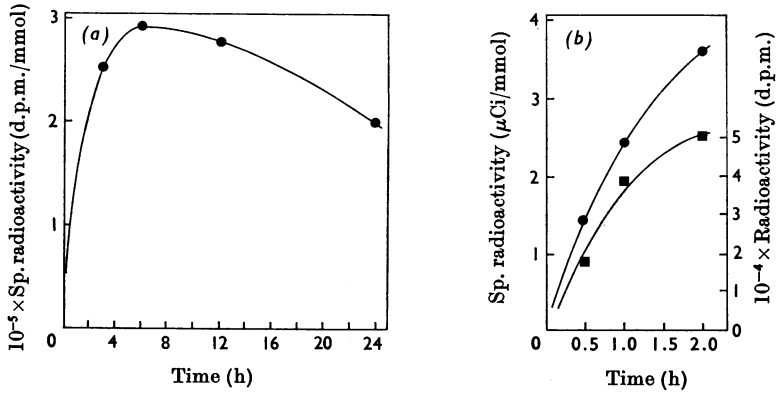


Fig. 2. Changes in the specific radioactivity of rosmarinic acid and the total radioactivity of ethanol-insoluble cinnamoyl esters in *Mentha*. Shoots were given a pulse-feeding of [^{14}C]phenylalanine for (*a*) 2h and (*b*) 0.5h and analysed at subsequent intervals. ●, Sp. radioactivity of rosmarinic acid; ■, total radioactivity of ethanol-insoluble esters.

Table 2. *Turnover of labelled rosmarinic acid readministered to* Mentha

Values in parentheses represent mg fed/g fresh wt. of plant material.

Compound fed	Amount fed (mg)	Radioactivity fed (d.p.m.)	Time fed (h)	Radioactivity in ethanol-soluble fraction (as % of compound fed)	Rosmarinic acid radioactivity (d.p.m./mmol)
Rosmarinic acid (caffeoyl-^{3}H)*	1.5 (0.33)	6.8×10^5	1	88	
	1.5 (0.29)	6.8×10^5	24	83	
	2.5 (0.45)	1.1×10^6	2	85	3.28×10^6
	2.5 (0.52)	1.1×10^6	24	82	2.86×10^6
Rosmarinic acid (DOPL-^{3}H)†	1.5 (0.36)	9.5×10^5	1	82	
	1.5 (0.33)	9.5×10^5	24	53	
	2.5 (0.42)	1.6×10^6	2	75	1.28×10^7
	2.5 (0.45)	1.6×10^6	24	55	8.30×10^6

* Caffeoyl moiety with greater than 96% of the ^{3}H, sp. radioactivity 73.5 μCi/mmol.
† DOPL moiety with greater than 98% of the ^{3}H, sp. radioactivity 102 μCi/mmol.

ever, no reason to believe from these preliminary experiments that either moiety of rosmarinic acid is contributing in a major way to lignin formation, as suggested for chlorogenic acid in other plants (Taylor & Zucker, 1966).

Further studies of the metabolism of rosmarinic acid and of its DOPL moiety will require much higher specific radioactivities in the ester and the use of ^{14}C to avoid the well-known difficulties with the ^{3}H label (Wang & Willis, 1965).

B.E.E. held a National Research Council Canada Scholarship. We acknowledge financial assistance by the National Research Council for this work.

REFERENCES

Austin, D. J. & Meyers, M. B. (1965). *Phytochemistry*, **4**, 425.

Bergmann, L., Thies, W. & Erdelsky, K. (1965). *Z. Naturf.* **206**, 1297.

Bray, H. G., Thorpe, W. V. & White, K. (1950). *Biochem. J.* **46**, 271.

El-Basyouni, S. Z. & Neish, A. C. (1966). *Phytochemistry*, **5**, 683.

El-Basyouni, S. Z., Neish, A. C. & Towers, G. H. N. (1964). *Phytochemistry*, **3**, 627.

Fritig, B. (1968). Ph.D. Thesis: University of Strasbourg.

Gamborg, O. L. (1967). *Can. J. Biochem.* **45**, 1451.

Gamborg, O. L. & Wetter, L. R. (1963). *Can. J. Biochem. Physiol.* **41**, 1733.

Harborne, J. B. (1966). *Z. Naturf.* **21**b, 604.

Harborne, J. B. & Corner, J. J. (1961). *Biochem. J.* **81**, 242.

Hiller, K. & Kothe, N. (1967). *Pharmazie*, **22**, 220.

Horhammer, L., Wagner, H. & Fritzsche, W. (1964). *Biochem. Z.* **339**, 398.

Ibrahim, R. K., Lawson, S. G. & Towers, G. H. N. (1961). *Can. J. Biochem. Physiol.* **39**, 873.

Kagan, J. (1966). *J. Am. chem. Soc.* **88**, 2617.

Kahnt, G. (1966). *Biol. Zbl.* **85**, 545.

Karrer, W. (1958). *Konstitution und Vorkommen der organischen Pflanzenstoffe*, pp. 964–965. Basle and Stuttgart: Birkhauser-Verlag.

Kojina, M., Minamikawa, T., Hyodo, H. & Uritani, I. (1969). *Pl. Cell Physiol., Tokyo*, **10**, 471.

Koukol, J. & Conn, E. E. (1963). *J. biol. Chem.* **236**, 2692.

Kovacs, P. & Jindra, A. (1964). *Experientia*, **21**, 18.

Leete, E. (1966). In *Biogenesis of Natural Compounds*, 2nd ed., chapter 17. Ed. by Bernfeld, P. Oxford: Pergamon Press Ltd.

Levy, C. C. & Zucker, M. (1960). *J. biol. Chem.* **235**, 2418.

McCalla, D. R. & Neish, A. C. (1959*a*). *Can. J. Biochem. Physiol.* **37**, 531.

McCalla, D. R. & Neish, A. C. (1959*b*). *Can. J. Biochem. Physiol.* **37**, 537.

MacLeod, N. J. & Pridham, J. B. (1963). *Biochem. J.* **88**, 45P.

Nair, P. M. & Vining, L. D. (1965). *Phytochemistry*, **4**, 401.

Neish, A. C. (1961). *Phytochemistry*, **1**, 1.

Ribereau-Gayon, P. (1965). *C. r. hebd. Séanc. Acad. Sci., Paris*, **260**, 341.

Rohringer, R. & Samborski, D. J. (1967). *A. Rev. Phytopath.* **5**, 77.

Rosa, N. (1966). Ph.D. Thesis: Dalhousie University, Halifax, N.S., Canada.

Runeckles, V. C. (1963). *Can. J. Biochem. Physiol.* **41**, 2249.

Runeckles, V. C. & Woolrich, K. (1963). *Phytochemistry*, **2**, 1.

Scarpati, M. L. & Oriente, G. (1958*a*). *Tetrahedron*, **4**, 43.

Scarpati, M. L. & Oriente, G. (1958*b*). *Ricerca scient.* **28**, 2329.

Scarpati, M. L. & Oriente, G. (1960). *Gazz. chim. ital.* **90**, 212.

Sondheimer, E. (1958). *Archs Biochem. Biophys.* **74**, 131.

Steck, W. (1968). *Phytochemistry*, **7**, 1711.

Taylor, A. O. (1968). *Phytochemistry*, **7**, 63.

Taylor, A. O. & Zucker, M. (1966). *Pl. Physiol., Lancaster*, **41**, 1350.

Wang, C. H. & Willis, D. L. (1965). *Radiotracer Methodology in Biological Science*, pp. 236–238. Clifton Heights, N.J.: Prentice-Hall Inc.

Wright, D., Brown, S. A. & Neish, A. C. (1958). *Can. J. Biochem. Physiol.* **36**, 1087.