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SHORT COMMUNICATION

THE 'NIH-SHIFT' DURING AROMATIC *ORTHO*-HYDROXYLATION IN HIGHER PLANTS

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Abstract—By feeding *ortho*-^{3}H cinnamic acid to *Melilotus* and *ortho*-^{3}H cinnamic and benzoic acids to *Gaultheria* it has been shown that *ortho*-coumaric acid biosynthesis involves an efficient migration and retention of the *ortho* proton while salicylic acid biosynthesis involves a much lower retention.

INTRODUCTION

THE PHENOMENON of proton migration from the site of biological aryl hydroxylations (the 'NIH-Shift') has been closely examined in animal and microbial systems[1, 2] but only to a small extent in plants. In higher plants the *para*-hydroxylation of cinnamic acid to form *p*-coumaric acid[3–6] has been studied and an efficient migration and retention (85%) of the *para*-proton has been demonstrated. Subsequent hydroxylations *ortho* to this *para*-hydroxyl have been shown to involve effectively no proton migration.[4–6]

The initial hydroxylation of aromatic compounds *ortho* to a side chain has been briefly examined in a mammalian microsomal system.[7] This may also be a common reaction in plant cells, notably in the biosynthesis of coumarins from cinnamic acid.[8] Furthermore, it is known that benzoic acid and phenylpyruvic acid can be hydroxylated to form salicylic acid[9] and *o*-hydroxyphenylacetic acid[10] respectively, in the latter case with a concomitant oxidative decarboxylation and sidechain migration.

The present work was undertaken to demonstrate the extent of proton retention during the biosynthesis of *o*-hydroxy cinnamic and benzoic acids in higher plants.

RESULTS AND DISCUSSION

Cinnamic acid-*ortho*-^{3}H, *ring*-1-^{14}C fed to *Melilotus alba* Desr. shoots was incorporated to a small extent into coumarin and more effectively into *o*-coumaric acid, which is in agreement with the results previously obtained by Kosuge and Conn.[8] The ratio of tritium to C-14 (T/C) in the purified products showed only a small decrease (Table 1) from the T/C ratio of the precursor, indicating a highly efficient migration of the *ortho*-proton

[1] G. GUROFF, J. W. DALY, D. M. JERINA, J. RENSON, B. WITKOP and S. UDENFRIEND, *Science* **157**, 1524 (1967).
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[5] N. AMRHEIN and M. H. ZENK, *Phytochem.* **8**, 107 (1969).
[6] W. R. BOWMAN, I. T. BRUCE and G. W. KIRBY, *Chem. Commun.* 1075 (1969).
[7] J. DALY and D. JERINA, *Arch. Biochem. Biophys.* **134**, 266 (1969).
[8] T. KOSUGE and E. E. CONN, *J. Biol. Chem.* **234**, 2133 (1959).
[9] S. Z. EL-BASYOUNI, D. CHEN, R. K. IBRAHIM, A. C. NEISH and G. H. N. TOWERS, *Phytochem.* **3**, 485 (1964).
[10] H. KINDL, *Europ. J. Biochem.* **7**, 340 (1969).

TABLE 1. THE MIGRATION AND RETENTION OF TRITIUM DURING *ortho*-HYROXYLATION OF CINNAMIC AND BENZOIC ACID LABELLED WITH TRITIUM IN RING POSITIONS 2 AND 6

Compound fed	Amount fed (μM)	T/C ratio	Compound isolated	T/C ratio	retention of tritium* Percent migration and
Cinnamic acid†	2·24	19 5	Coumarin	17 3	78
			o-coumaric acid	18·8	92
Cinnamic acid‡	2·12	27·4	Salicylic acid	17·3	26
Cinnamic acid‡	2·12	21·6	Salicylic acid	14·6	35
Benzoic acid‡	1·57	3·3	Salicylic acid	1·9	16

* Calculated on the basis of complete retention of label at one of the two ring positions, 2 or 6.
† *Melilotus alba* Desr.
‡ *Gaultheria procumbens* L.

to a neighbouring ring position. Reported retentions of deuterium during *ortho*-hydroxylation of various substrates by mammalian microsomal preparations ranged from 24 to 60% depending upon the substrate.[7]

When the same precursor was administered to young *Gaultheria procumbens* L. leaves, however, and salicylic acid subsequently isolated, the degree of retention of the *ortho*-proton was strikingly lower (Table 1). This contrast with the result in *M. alba* prompted an examination of benzoic acid as an intermediate in the biosynthesis of salicylic acid from cinnamic acid. Benzoic acid-*ortho*-^{3}H, *ring*-1-^{14}C, fed to *G. procumbens* was efficiently incorporated into salicylic acid and the extent of proton retention was again very low (Table 1). It seemed possible that the low values might result from a carboxyl migration from the site of hydroxylation, which might be expected to displace the *ortho*-proton. Degradation of a salicylic acid-^{14}C sample obtained from *G. procumbens* leaves fed benzoic acid-*ring*-1-^{14}C showed however that all the radioactivity could be recovered in the carbon dioxide derived[12] from carbons-1,3 and 5 of the ring of salicylic acid (Table 2), which eliminates the possibility of a carboxyl shift from the site of hydroxylation.

TABLE 2. DEGRADATION OF SALICYLIC ACID-^{14}C BIOSYNTHESIZED FROM BENZOIC ACID-*ring*-1-^{14}C IN *Gaultheria procumbens* L. LEAVES

Compound	Carbons of salicylic acid	Specific act. dis/min/μM	Sp. act. as per cent of sp. act. of salicylic acid
Salicylic acid	(C_{1-7})	370	100
Picric acid	(C_{1-6})	391	106
Bromopicrin	($C_{1,3,5}$)	not measured	—
Carbon dioxide	($C_{1,3,5}$)	133·5	108*

* Corrected for a yield of 3 mole CO_2 from each mole of salicylic acid.

[11] G. H. N. TOWERS, A. TSE and W. S. G. MAASS, *Phytochem.* **5**, 677 (1970).
[12] H. SIMON and H. G. FLOSS, in *Bestimmung der Isotopenverteilung in markierten Verbindungen*, p. 124, Springer–Verlag, Berlin (1967).

Whether the hydroxylation patterns of the hydroxybenzoic acids in plants have been established at the cinnamic acid level before β-oxidation, or by hydroxylation of another benzoic acid after β-oxidation is still not clear[9, 13, 14] and both routes are probably used in plants with one or the other playing a dominant role depending on conditions or species. If the proton retention observed in *o*-coumaric acid biosynthesis in this study can be taken as typical for *ortho*-hydroxylation of cinnamic acid in higher plants, the present results indicate that the main route of formation of salicylic acid in young *Gaultheria* tissue is cinnamic acid → benzoic acid → salicylic acid, because both cinnamic and benzoic acid are incorporated into salicylic acid with similar low *ortho*-proton retention (Fig. 1).

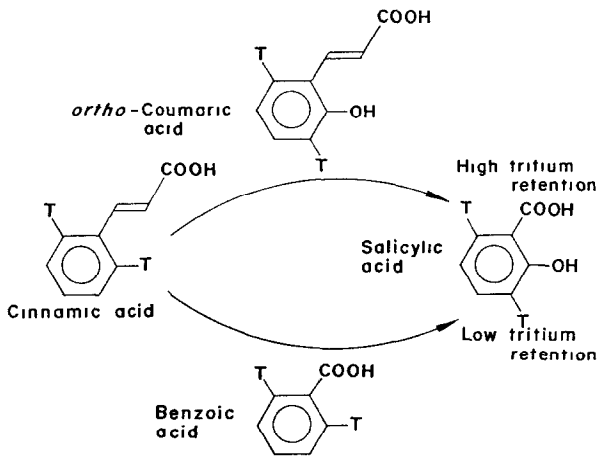

FIG. 1. THE POSSIBILITIES FOR SALICYLIC ACID BIOSYNTHESIS FROM CINNAMIC ACID IN *Gaultheria procumbens*.*

*The tritium retention values observed when *ortho*-^{3}H cinnamic acid was used as a precursor (26–35%) suggest that most, if not all, of the salicylic acid is being formed by the benzoic acid route.

EXPERIMENTAL

Plant material. Melilotus alba Desr. and *Gaultheria procumbens* L. were grown in the University green house at 23° and under natural lighting.

Preparation and feeding of radioactive compounds. Cinnamic acid-*ring*-1-^{14}C was prepared from DL-phenylalanine-*ring*-1-^{14}C (New England Nuclear) by use of a phenylalanine ammonia-lyase preparation from illuminated buckwheat seedlings.[15] Similarly, cinnamic acid-*ortho*-^{3}H was prepared from DL-phenylalanine-*ortho*-^{3}H which had been prepared by L. Nover, Martin Luther Universität, Halle–Wittenberg.[16]

Benzoic acid-*ortho*-^{3}H was prepared by mild $KMnO_4$ oxidation of cinnamic acid-*ortho*-^{3}H. Benzoic acid-*ring*-1-^{14}C was purchased from The Radiochemical Centre, Amersham. All compounds used were purified by paper chromatography and all measurements of radioactivity were made by liquid scintillation counting.

Melilotus shoots (*ca.* 2 g, 4-week-old) were allowed to take up an aq. solution of double-labelled cinnamic acid by transpiration and subsequently placed in distilled water to complete the 28-hr metabolic period under natural lighting.

Two-week-old *Gaultheria* leaves were sliced into 4 mm wide sections and vacuum infiltrated with distilled water. The sections (*ca.* 500 mg) were then floated on 5 ml of an aqueous solution of the radioactive compound in a Petri dish under continuous illumination for 24-hr.

Isolation and purification of coumarin and o-*coumaric acid.* Coumarin was isolated from the *Melilotus* shoots as described by Kosuge and Conn.[8] After addition of carrier the ether extract was chromatographed

[13] H. GRISEBACH and K.-O. VOLLMER, *Z. Naturforsch.* **19b**, 781 (1964).
[14] M. H. ZENK and G. MÜLLER, *Z. Naturforsch.* **19b**, 398 (1964).
[15] N. AMRHEIN and M. H. ZENK, *Z. Pflanzenphysiol.* in press.
[16] L. NOVER and M. LUCKNER, *FEBS Letters* **3**, 292 (1969).

on paper in *iso*propanol–H_2O (1:4) and then on silica gel plates in *n*-hexane–EtOAc (72:29), and toluene–acetone (95:5). The coumarin band was eluted with 80% ethanol.

The aq. solution remaining after ether extraction for coumarin isolation was adjusted to pH 5·5 and treated overnight with β-glucosidase. Ether extraction of the hydrolysate yielded *o*-coumaric acid which was purified on silica gel plates using benzene–HOAc (8:2) and toluene–$CHCl_3$–acetone (40:35:25), followed by paper chromatography using 4% formic acid.

The identity of the compound was checked by R_fs, the UV spectrum and by methylation with diazomethane which yielded a product chromatographically identical with that from the methylation of authentic *o*-coumaric acid. Finally the *o*-coumaric acid was crystallized to constant specific activity with carrier without altering the T/C ratio.

Isolation and Purification of Salicylic Acid

The *Gaultheria* leaf sections were extracted with hot 80% ethanol until colourless. The ethanol extract was reduced to a small volume, taken up in hot water and filtered. The filtrate was then chromatographed on a small column of Avicel developed with 2% formic acid. The fractions containing gaultherin (gentiobioside of methyl salicylate) were evaporated to a small volume Half was used to obtain pure gaultherin by paper chromatography using *n*-BuOH–HOAc–H_2O (4:1:2·2), *iso*-ProH–NH_4OH–H_2O (8:1·1) and 2% formic acid. The other half was hydrolyzed with NaOH and then with HCl, followed by ether extraction. The salicylic acid in the ether extract was purified by paper chromatography using benzene–HOAc–H_2O (10·7:3, org. phase), *iso*-ProH–NH_4OH–H_2O (8:1:1) and 2% formic acid The T/C ratios of the salicylic acid and gaultherin were always in good agreement and usually changed little during the purification.

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