Biosynthetic Incorporation of [β-^{14}C; 3,5-$^{2}H_2$; 4-^{3}H]Cinnamic Acid into Capsaicin and Norpluviine: Lack of an Apparent Isotope Effect following an NIH Shift

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Summary Biosynthetic experiments with [4-^{3}H]-, [4-^{3}H; 3,5-$^{2}H_2$]- and [3-^{3}H]cinnamic acid, using *Capsicum annuum* and "Texas" daffodils, have shown that hydroxylation at C-4 involves migration of hydrogen to the neighbouring carbon (the "NIH shift") and that the aromatisation step following migration involves no apparent isotope effect and is presumably stereospecific and enzymically controlled.

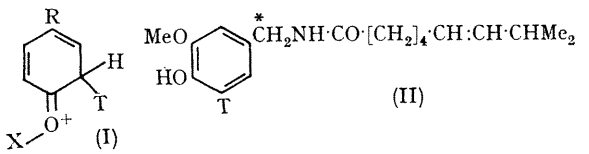
BIOLOGICAL hydroxylation[1] of aromatic substrates can involve a 1,2 migration of hydrogen to neighbouring carbon (the "NIH shift"). High retentions of tritium have been observed for the conversions of [4-^{3}H]phenylalanine[2] into tyrosine (>90%) and of [4-^{3}H]cinnamic acid[3] into *p*-coumaric acid (85%), the tritium appearing *ortho* to the phenolic hydroxy-groups of the products. In contrast, hydroxylation *ortho* to an existing hydroxy-group generally

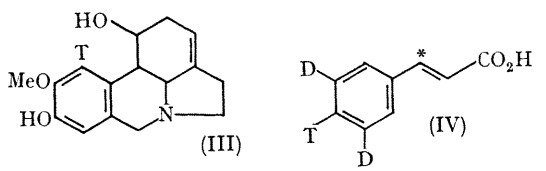
involves loss of labelled hydrogen from that position.[1,4] A survey of published work indicates[1] that oxygenation of the substrate, possibly to form an arene oxide,[5] is followed by migration of hydrogen (or tritium) to give an intermediate (I) which then spontaneously loses hydrogen or tritium to yield a phenol. The observed high retention of tritium is reasonably explained assuming the operation of a kinetic isotope effect in the final step. Work[6] on the acid-catalysed exchange reactions of phenolic ethers, which involve intermediates of the type (I), has established an isotope effect, k_H/k_T = *ca.* 17, for hydrogen loss during aromatisation. This value is large enough adequately to explain the extent of tritium retention during biological hydroxylation. We report experiments designed to measure accurately the isotope effect involved in the final step of the hydroxylation of cinnamic acid in higher plants.

Incorporation[a] *of cinnamic acids into capsaicin and norpluviine*

Labelling pattern of precursor	$^3H/^{14}C$ ratio Precursor	$^3H/^{14}C$ ratio Capsaicin	During biosynthesis	% Loss of ^3H Calc.[b]	% Loss of ^3H After exchange
[β-^{14}C; 4-^{3}H]	8·24	3·95	52	52·8	96
[β-^{14}C; 4-^{3}H; 3,5-$^2H_2$]	7·45	3·70	50	64·7	89
	Precursor	Norpluviine			
[β-^{14}C; 4-^{3}H]	7·64	3·92	49	52·8	3·5
[β-^{14}C; 4-^{3}H; 3,5-$^2H_2$]	3·69	1·92	48	64·7	1·9
[β-^{14}C; 3-^{3}H]	8·34	2·35	72	51·4	2·0

[a] Incorporations all within 0·06—0·09%.
[b] Calculated (see text) assuming k_H/k_T = 17 and k_D/k_T = 2·4.





The aromatic, C_6–C_1 units of capsaicin[7] (II) and norpluviine[8] (III) are known to be derived biosynthetically from phenylalanine *via* cinnamic acid and hydroxylated cinnamic acids. [β-^{14}C; 4-^{3}H]Cinnamic acid and [β-^{14}C; 4-^{3}H; 3,5-$^2H_2$]cinnamic acid (IV)† were fed in parallel to *Capsicum annuum* plants. The $^3H/^{14}C$ ratios of precursors and the derived capsaicin are tabulated. Base-catalysed exchange of the capsaicin specimens showed[7] that essentially all (accurate measurement of residual tritium in the presence of ^{14}C is difficult) the tritium was, as expected, *ortho* to the phenolic hydroxy-group. Retention of *ca.* 50% of tritium during the metabolism of [4-^{3}H]cinnamic acid is consistent with (a) *para*-hydroxylation involving virtually complete migration and retention of tritium, and (b) a second hydroxylation at one of two equivalent positions causing loss, without migration, of half the remaining tritium. However, the same result was observed with the deuteriated precursor (IV). Tritium loss, without enzymic catalysis, from an intermediate of the type (I) would be governed in the first experiment by an H/T isotopic effect and in the second experiment by a much smaller D/T isotope effect. The difference in observed retentions would have been easily detected experimentally (see Table for typical values). The lack of any apparent isotope effect therefore excludes an intermediate (I) unless hydrogen loss is stereospecific and, presumably, enzymically controlled.

Similar results were obtained for the biosynthesis of norpluviine (III) in "Texas" daffodils. Again, *ca.* 50% retention of tritium was observed for both the undeuteriated and deuteriated precursors. Base-catalysed exchange of the norpluviine showed,[9] as expected, no significant amounts of tritium *ortho* to the phenolic hydroxy-group. Confirmation of these results was obtained by feeding [β-^{14}C; 3-^{3}H]cinnamic acid. *para*-Hydroxylation would now cause migration, with retention, of *hydrogen* to carbon bearing either tritium or hydrogen. Half of the tritium would therefore be lost and half the remainder lost during the second hydroxylation. The observed loss (72%) agrees quite well with the predicted (75%) value and is clearly different from the 51% loss predicted assuming control solely by an isotope effect.

Our findings can be reconciled most economically with earlier work by the following assumptions. (a) Aromatic substrates are attacked by an oxygenase to give an arene oxide. (b) A second enzyme, an isomerase, converts the arene oxide into a phenol with retention of the migrating hydrogen and loss of hydrogen from carbon *ortho* to the point of attack. An intermediate (I) could be involved providing hydrogen loss therefrom is enzymically controlled and thereby stereospecific. (c) The purified or partially purified enzyme systems used in earlier work[1,2] contained the oxygenase but lacked the isomerase, whereas our intact plants contained both. In the absence of the isomerase, rearrangement of the arene oxide could still take place but retention of a migrating tritium would be governed by an isotopic effect. The recent report[5] of the non-enzymic

† The acid contained >95% D_2; ^{3}H and ^{14}C were present in low isotopic abundance. The synthesis ensured that all tritiated species were dideuteriated; the ^{14}C labelled species contained neither tritium nor deuterium. The position of tritium in the precursors was confirmed by conversion into ethyl 4-nitrocinnamate.

conversion of toluene oxide into *p*-cresol under "physiological conditions" elegantly demonstrates this possibility.

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