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DEGRADATION OF AROMATIC COMPOUNDS BY STERILE PLANT TISSUES

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Abstract—The ability of plant tissues to degrade aromatic rings (of phenylalanine, cinnamic acid, tryptophan) in the absence of micro-organisms has been established using plant tissue cultures (*Ruta, Melilotus*).

INTRODUCTION

MICRO-ORGANISMS readily degrade aromatic compounds of all types, oxidatively,[1] or less commonly reductively.[2] The initial aliphatic products of ring-fission are eventually metabolized to carbon dioxide. Higher plants accumulate large quantities of aromatic compounds, principally phenolics, ranging in structure from simple phenols to polymers such as lignins. Some of these compounds, notably those possessing *ortho*-dihydroxyls, are known to be ring-cleavage substrates in microbial metabolism.[1]

The ability of higher plants to degrade the aromatic structures which they have synthesized has received little attention. Tracer studies have been hampered by the scarcity of aromatic ring-^{14}C compounds and by the problem of eliminating the involvement of microbial metabolism. Despite this, a number of reports have appeared describing the ability of plants to split the aromatic ring of catechins, an "A"-ring-labelled chalcone,[3] 3,4-dihydroxyphenylalanine (DOPA),[4] tyrosine,[5] phenylalanine,[6] toluene,[7] benzene[7,8] or phenol.[9] In no case could the absence of micro-organisms from the experimental system be assured. This leaves some doubt concerning the validity of the results, a problem which has recently appeared in studies of tryptophan metabolism in plants.[10] In view of the potential importance of ring-fission reactions in plant phenolic metabolism an unequivocal demonstration of the ability of plant tissues to degrade aromatic rings to carbon dioxide is desirable. With the recent availability of a number of aromatic ring-^{14}C compounds, a study of their degradation by aseptic plant tissues was undertaken.

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RESULTS AND DISCUSSION

Preliminary experiments carried out at Prairie Regional Laboratory* showed that suspension cultures of wheat, soybean and mung bean tissues produced small amounts of $^{14}CO_2$ from phenylalanine-*ring*-^{14}C. This work was later extended to tissue cultures of *Ruta graveolens* and *Melilotus alba*,[11] both of which were shown to be capable of degrading the aromatic ring of phenylalanine (Table 1). Earlier work with shoots of *Hordeum vulgare* in a non-sterile system gave similar results.[6] Since phenylalanine is a known precursor to the phenolic acids in plants, it was possible that phenylalanine was being degraded after conversion to dihydroxycinnamic acid or benzoic acid. Cinnamic acid-*ring*-^{14}C, prepared using phenylalanine ammonia lyase,[12] was tested as substrate. The *Melilotus* tissue degraded the ring of

TABLE 1. METABOLISM OF DL-PHENYLALANINE, CINNAMIC, SALICYLIC AND BENZOIC ACIDS BY *Ruta* AND *Melilotus* OVER 7 days

Compound fed	Tissue	Activity fed (μc)	% Activity taken up	% Activity recovered in CO_2*
DL-Phenylalanine-*ring*-1-^{14}C, 3·44 mc/mM	*Melilotus*	2·8	86	0·14
		7·5	79	0·14
	Ruta	2·5	94·9	0·31
		4·2	95·9	0·17
		5·0	95·3	0·12
Cinnamic acid-*ring*-1-^{14}C, 3·44 mc/mM	*Melilotus*	1·2	84	0·04
		3·5	71·1	0·39
	Ruta	2·1	39·9	N.D.†
		3·5	94	N.D.
DL-Phenylalanine-2-^{14}C, 2·5 mc/mM	*Ruta*	2	88	1·1
Cinnamic acid-2-^{14}C, 50 mc/mM	*Melilotus*	1	73·5	3·8‡
	Ruta	2	88·2	0·71
Benzoic acid-*ring*-U-^{14}C, 45 mc/mM	*Ruta*	5	96·7	N.D.†
	Ruta	5	96·5	N.D.
	Melilotus	3	65·9	N.D.
Salicylic acid-*ring*-U-^{14}C, 0·95 mc/mM	*Ruta*	5	82·1	N.D.
	Melilotus	3	88·2	N.D.

* As percentage of activity taken up.
† Not detectable, less than 0·02%
‡ Incubation for 4 days.

cinnamic acid to carbon dioxide at least as well as that of phenylalanine (Table 1). *Ruta*, on the other hand, appeared to be unable to degrade cinnamic acid. The *Ruta* tissue has an active cinnamate metabolism, however, since it accumulates rutin and several coumarins.[13] This may indicate that in some plants phenylalanine can be degraded through a pathway involving non-cinnamate structures such as the hydroxyphenylacetic acids.[14]

In higher plants *beta*-oxidation of the cinnamic acids yields benzoic acids,[15] which could serve as ring-cleavage substrates. Phenylalanine-2-^{14}C and cinnamic acid-2-^{14}C administered

* National Research Council of Canada, Saskatoon, Canada.

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to these tissues both gave rise to $^{14}CO_2$ (Table 1) suggesting that some of the phenylpropanoid compounds are undergoing *beta*-oxidation. Neither benzoic acid nor salicylic acid were degraded by the *Ruta* and *Melilotus* tissues, however (Table 1). Salicylic acid is decarboxylated in some plants to yield catechol,[16] a frequent intermediate in ring-cleavage pathways in microbial metabolism.[1] Aseptically grown tomato seedlings produced no detectable $^{14}CO_2$ however when they were exposed to catechol-^{14}C.[17]

Tyrosine

The rapidity with which the label from tyrosine administered to plants appears in non-aromatic compounds has been noted in several studies.[5,18,19] In one case, the degradation apparently included ring-fission.[5] Although ring-^{14}C tyrosine was unavailable we circumvented this problem by a comparison of the extent of $^{14}CO_2$ release from identical cultures containing either tyrosine-U-^{14}C or tyrosine-1,2,3-^{14}C (same total and specific activity). Both *Ruta* and *Melilotus* degraded tyrosine to a remarkable extent, but the ratio of 3:1 for

TABLE 2. DEGRADATION OF TYROSINE AND DOPA BY *Ruta* AND *Melilotus* OVER 7 days

Compound fed	Tissue	Activity fed (μc)	% Activity taken up	% Activity recovered in CO_2*
DL-Tyrosine-1,2,3-^{14}C, 5·14 mc/mM	*Ruta*	2·16	n.m.†	19·69
L-Tyrosine-U-^{14}C, 5·14 mc/mM	*Ruta*	2·0	n.m.	6·65
DL-Tyrosine-1,2,3-^{14}C, 5·14 mc/mM	*Ruta*	1	93·9	19·20
L-Tyrosine-U-^{14}C, 5·14 mc/mM	*Ruta*	1	92·3	4·35
DL-Tyrosine-1,2,3-^{14}C	*Melilotus*	1	81	7·07
L-Tyrosine-U-^{14}C	*Melilotus*	1	83	2·58
DL-Tyrosine-2-^{14}C, 50 mc/mM	*Ruta*	1	n.m.	21·00
DL-Tyrosine-3-^{14}C, 6·85 mc/mM	*Ruta*	1	n.m.	10·30
DL-Tyrosine-2-^{14}C, 0·90 mc/mM	*Ruta*	1	93·9	17·35
DL-Tyrosine-3-^{14}C, 0·90 mc/mM	*Ruta*	1	94·8	12·30

* As percentage of activity taken up.
† n.m. not measured: activity in CO_2 is percentage activity fed.

$^{14}CO_2$ from tyrosine-1,2,3-^{14}C:$^{14}CO_2$ from tyrosine-U-^{14}C was not exceeded by *Ruta* (Table 2). The slight increase in the ratio observed with *Melilotus* may not be significant. Thus, it appeared that no substantial ring-fission of tyrosine was occurring in these tissues. This method is not sufficiently sensitive to detect small amounts of ring-cleavage, however.

When tyrosine labelled in different side-chain positions was used as substrate, it was obvious that all the side-chain was being lost in the degradation (Table 2). An attempt was made to determine the route of degradation. DOPA-2-^{14}C yielded less than 3 per cent of the $^{14}CO_2$ released from tyrosine-2-^{14}C, making it unlikely that DOPA is a major intermediate in the degradation. On the other hand, indirect evidence suggested that homogentisic acid might be involved. Addition of homogentisic acid to cultures of *Melilotus* metabolizing tyrosine-2-^{14}C reduced the release of $^{14}CO_2$ by 30 per cent. The same concentration of homogentisic acid had no effect on the release of $^{14}CO_2$ from glucose-U-^{14}C. Homogentisic

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acid administered to *Melilotus* tissue together with tyrosine-2-^{14}C was found to contain appreciable label when extracted from the cells and isolated chromatographically. Homogentisic acid is a well-known ring-fission substrate in animals and micro-organisms. Its known role in plant metabolism is that of an intermediate in plastoquinone biosynthesis,[20] but it has been suggested as a ring-cleavage substrate during tyrosine degradation in *Pyrus* leaf discs.[5] The present work appears to reinforce that possibility.

Tryptophan

Tryptophan–*benzene-ring*-^{14}C is taken up less efficiently than the other amino acids by *Ruta* and *Melilotus* but was degraded to carbon dioxide by both tissues (Table 3). In micro-organisms tryptophan degradation leads to ring-fission through either anthranilic acid,[21] 3-hydroxyanthranilic acid[22] or kynurenate derivatives.[23] Whether any or all of these compounds are involved in tryptophan degradation in plant tissues is unknown.

It has thus been established that plants are able to cleave the aromatic ring of both phenylpropanoid and indole compounds. Whether the immediate ring-cleavage substrates are *ortho*-dihydroxy compounds such as caffeic acid, or *para*-dihydroxy compounds such as homogentisic acid, will require more detailed work. The use of plant tissue cultures provides a convenient, uniform source of actively growing plant tissue completely free from micro-organisms. The low levels of ring-cleavage detected with these tissues may not be typical of the levels occurring in intact plants. This possibility could be examined by use of aseptically grown plants and completely aseptic techniques.

TABLE 3. OXIDATION OF DL-TRYPTOPHAN-*benzene ring*-U-^{14}C TO $^{14}CO_2$ BY *Ruta* AND *Melilotus* OVER 7 days

Tissue	Activity fed (μc)	% Activity taken up	% Activity recovered in CO_2*
Ruta	1	67	0·22
	1	70	0·26
	4	89	0·03
Melilotus	2	51·5	0·23

* As percentage of activity taken up.

EXPERIMENTAL

Plant Material

Inocula for tissue cultures of *Ruta graveolens* and *Melilotus alba* were obtained from Dr. O. L. Gamborg and maintained on standard media[11] B5 (*Ruta*) and B5C2* (*Melilotus*) both as callus and as liquid suspension cultures. The stock cultures were grown at 23° under continuous fluorescent lighting (9000 lx) and required subculturing at 2–4-week intervals. Under these conditions the *Ruta* grew in liquid suspension as expanding lumps of multicellular green tissue, while the *Melilotus* grew as a pale-brown suspension of single cells and mall clusters of cells.

* B5 medium supplemented with 2 g/l. of N–Z Amine Type A casein hydrolysate (Sheffield Chemical, Norwich, N.Y., U.S.A.).

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Radioactive Compounds

DL-Phenylalanine-2-^{14}C, DL-DOPA-2-^{14}C, DL-tyrosine-1-^{14}C, DL-tyrosine-3-^{14}C, and DL-phenylalanine-*ring*-1-^{14}C were obtained from New England Nuclear, Boston, Mass. DL-Tyrosine-2-^{14}C, DL-tryptophan-*benzene-ring*-U-^{14}C, L-tyrosine-U-^{14}C and benzoic acid-*ring*-U-^{14}C were obtained from the Radiochemical Centre, Amersham, England. Cinnamic acid-2-^{14}C was purchased from International Chemical and Nuclear Corp., City of Industry, California, and salicylic acid-*ring*-U-^{14}C was obtained from Mallinckrodt Nuclear, Orlando, Florida.

Cinnamic acid-*ring*-1-^{14}C was prepared from phenylalanine ring-1-^{14}C by use of phenylalanine ammonia-lyase. The enzyme was prepared from *Ustilago hordei* E_3 and purified to the end of the first ammonium sulphate fractionation.[12] The cinnamic acid obtained was purified by TLC.

All compounds were made up in aqueous solution and sterilized by Millipore filtration.

Detection of Ring-cleavage

A feeding flask consisted of a cotton-stoppered 250 ml Erlenmeyer flask with a center well. To begin an experiment, a heavy inoculum of tissue and an aliquot of a radioactive compound were added to the 50 ml of medium. After 24 hr incubation on a gyrotary shaker (100 rev/min, 22–23°, continuous low light), the center well was charged with 2 ml 5 N KOH and a sterile filter paper wick (4 × 5 cm, Whatman No. 50). In early experiments the CO_2 trapping was carried on continuously, changing the KOH and wick at 24-hr intervals. This was found to allow appreciable losses of CO_2 through the stopper, however. Later experiments (most of those reported) were run by trapping CO_2 for 24 hr with a tight foil cap over the stopper, followed by 24 hr incubation without trapping. There was no indication that sealing the flask for 24 hr depressed growth of the cultures. In the course of an experiment the *Ruta* tissue increased in mass about 3×, while the mass of *Melilotus* increased from 5 to 10×.

The KOH and wick, aseptically removed from a feeding flask, were placed in a 125-ml side-arm flask. After connecting this to an air-flushing system the CO_2 was regenerated from the carbonate by addition of 2 ml of 50% lactic acid. The system was then flushed with air (*ca.* 60 ml/min) for 20 min and the CO_2 trapped in 10 ml of 2-phenylethylamine. Aliquots of the 2-phenylethylamine were counted in duplicate by liquid scintillation counting (to a minimum of 1000 counts/min). Tests with $Na_2{}^{14}CO_3$ in the medium of the feeding flask and 16 hr trapping after acidification showed that the recovery of activity in the complete procedure was 70–80 per cent.

At the end of a feeding experiment the residual radioactivity in the medium was determined after removal of the tissue by filtration.

Microbial Contamination

Contamination of feeding experiments, stock cultures or radiochemical solutions was checked by plating the tissue or solution on two media, yeast extract (1%)–glucose (1%) and malt extract (4·5%). The plates were incubated at 33° for 2 days and then 23° for 3 days.

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