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THE METABOLISM OF FUMARATE BY PEACH, APPLE AND PRIVET ROOTS

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Abstract—Excised roots of peach, apple and privet respired the bulk (40–73%) of absorbed fumarate-1,4-^{14}C to CO_2 by 320 min. On the other hand, only 12% of the absorbed fumarate-2,3-^{14}C was respired to CO_2 by peach roots, while privet roots converted less than 1%. The fate of fumarate-2,3-^{14}C was greatly dissimilar among the three species; peach roots incorporated 50% of the absorbed label into protein, apple 33% and privet 10%. Privet roots contained the most, and peach roots the least, ^{14}C in organic acids. The rapid metabolism of fumarate-2,3-^{14}C to glutamate and aspartate and the large incorporation of these amino acids into protein by the three species indicates the presence of enzyme systems important in amino acid synthesis in the roots of woody plants, and the labelling pattern suggests that aspartase is not physiologically important in these tissues.

INTRODUCTION

ZIMMERMAN[1] and Joy[2] have shown that photosynthetic assimilates translocated downward in phloem tissues can be used for the synthesis of amino acids in roots; and the synthesis of amino acids from keto acids has been reported in roots by Kursanov.[3] Some of the amino acids are used in root growth and some are found in the translocation stream.

In many species of woody plants,[4] including apple,[5] glutamate, aspartate, and their amides are important translocatory forms of nitrogen and it can be assumed then that these substances are physiologically important in upward nitrogen transport. When α-ketoglutarate was fed to roots of woody plants,[6] glutamate was found to accumulate earlier than aspartate, and it was concluded that glutamate was formed either by glutamic dehydrogenase or by transamination, and that aspartate could be formed by aspartase. The experiments reported here describe the metabolism of fumarate-1,4-^{14}C and fumarate-2,3-^{14}C in excised roots of three species of woody plants, and suggest that aspartase activity is not physiologically significant in these roots.

RESULTS

The release of $^{14}CO_2$ from fumarate-2,3-^{14}C and fumarate-1,4-^{14}C is shown in Fig. 1. Initially, little ^{14}C was lost as CO_2 from either of the labelled fumarates. By 320 min, however, 73% of the absorbed label from fumarate-1,4-^{14}C was respired to $^{14}CO_2$ by peach roots. Apple and privet roots also converted the bulk of the absorbed label to $^{14}CO_2$. This is in contrast to $^{14}CO_2$ released from fumarate-2,3-^{14}C where apple roots released only 12% of the

[1] M. H. ZIMMERMAN, *Ann. Rev. Plant Physiol.* **11**, 167 (1960).
[2] K. W. JOY, *J. Exptl Botany* **18**, 140 (1967).
[3] A. L. KURSANOV, 8th Intern. Congress of Soil Science, Bucharest, Romania, **4**, 1069 (1964).
[4] E. G. BOLLARD, *Australian J. Biol. Sci.* **10**, 288 (1957).
[5] N. H. OZEROL and J. S. TITUS, *Proc. Am. Soc. Hort. Sci.* **93**, 7 (1968).
[6] J. S. TITUS, W. E. SPLITTSTOESSER and P. SPENCER, *Plant Physiol.* **43**, 619 (1968).

label as $^{14}CO_2$ and privet roots less than 1%. Subsequent experiments were conducted with fumarate-2,3-^{14}C.

In excised peach roots the label spread from fumarate-2,3-^{14}C into acids of the citric acid cycle, CO_2, insoluble residue and amino acids (Table 1). All of the components of these fractions continued to increase in label over the duration of the experiment, except fumarate which remained relatively constant. By 320 min, approximately 50% of the absorbed label was found in the insoluble residue, 5% in CO_2, glutamate, and fumarate, and about 10% in each of the malate, citrate, and aspartate fractions. Although only 5% of the absorbed ^{14}C was in glutamate, the bulk of the ^{14}C in the "all other" amino acid fraction after 160 min was in glutamine, and the amount of label in aspartate and asparagine together approximated that in glutamate and glutamine.

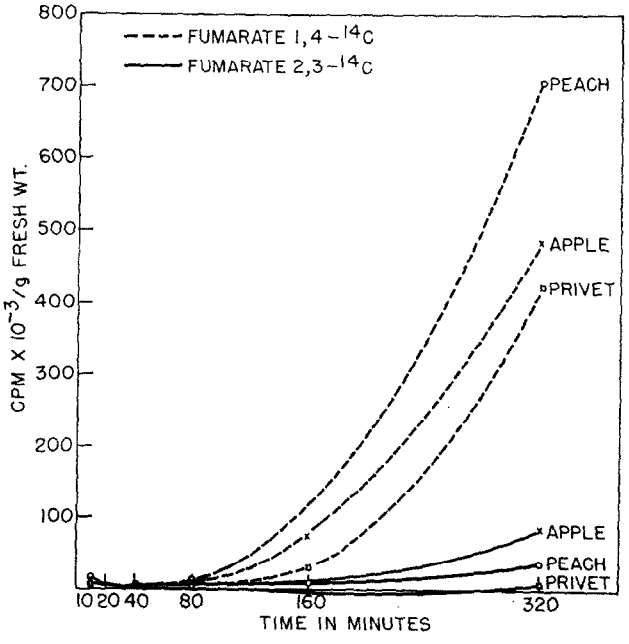

FIG. 1. THE RELEASE OF $^{14}CO_2$ FROM FUMARATE-^{14}C BY PEACH, APPLE AND PRIVET ROOTS.

Acid hydrolysis of the insoluble residue from peach roots indicated that glutamate, aspartate, and their amides were the principal labelled products. The carbon skeletons of glutamate and aspartate, both as free acids and their amides and incorporated into protein, accounted for 70% of the absorbed ^{14}C by 320 min.

The carbon from fumarate appeared in aspartate and glutamate as early as 10 min (Table 1). Shorter experimental times of 3 and 6 min were used in an attempt to determine which of these amino acids became labelled first. As early as 3 min, both glutamate and aspartate were labelled and these two amino acids contained 98% of the ^{14}C found in compounds other than fumarate-2,3-^{14}C.

The metabolism of fumarate-2,3-^{14}C by excised apple roots is shown in Table 2. Of the three species studied, apple tissue released the most label as $^{14}CO_2$ by 320 min, although little ^{14}C appeared in CO_2 before this time. Initially the bulk of the label from fumarate appeared in malate and by 320 min malate contained 33% of the absorbed ^{14}C. Citrate and succinate

also accumulated label; and ^{14}C appeared rapidly and accumulated steadily in glutamate, and also in aspartate but at a slower rate throughout. However, as in peach roots, both

TABLE 1. THE METABOLISM OF FUMARATE-2,3-^{14}C BY PEACH ROOTS

Fraction	Min incubated					
	10	20	40	80	160	320
	cpm × 10^{-3}/g fr. wt.					
CO_2	0·4	0	0·3	0·5	2·6	30·2
Amino acids						
Glutamate	1·0	2·3	4·1	8·3	10·9	27·3
Aspartate	1·3	2·9	6·8	6·1	15·8	60·1
All others	0·4	0·6	1·9	1·5	8·3	28·8
Organic acids						
Citrate	0·5	0·8	2·8	2·4	12·9	53·9
Malate	1·5	2·4	8·8	7·7	14·4	55·5
α-Ketoglutarate	0	0	1·1	0	1·3	3·2
Succinate	0·2	0	1·4	0	0·6	2·4
Fumarate	10·7	20·5	13·3	27·7	28·9	26·4
Sugars	0·2	0·1	0·6	0·5	1·2	2·3
Insoluble residue	4·5	3·7	11·4	22·0	74·5	291·3
Total ^{14}C absorbed	20·7	33·3	52·5	76·7	171·4	581·4

TABLE 2. THE METABOLISM OF FUMARATE-2,3-^{14}C BY APPLE ROOTS

Fraction	Min incubated					
	10	20	40	80	160	320
	cpm × 10^{-3}/g fr. wt.					
CO_2	0	0·2	0·2	0·6	0·2	86·0
Amino acids						
Glutamate	7·3	8·1	17·0	21·6	30·0	40·4
Aspartate	2·0	3·6	5·5	8·7	16·8	24·8
All others	2·7	3·3	6·0	8·8	12·8	17·3
Organic acids						
Citrate	0·8	2·7	3·0	11·9	8·2	26·0
Malate	14·3	18·5	31·5	57·3	129·2	228·0
α-Ketoglutarate	0·6	0·4	0·5	1·1	0	0
Succinate	0·9	2·4	3·4	3·2	4·9	8·6
Fumarate	21·0	15·3	17·4	34·6	21·3	26·0
Sugars	0·5	0·6	1·0	1·5	3·1	5·1
Insoluble residue	5·2	6·5	13·6	25·6	82·9	223·0
Total ^{14}C absorbed	55·3	61·6	99·5	174·9	309·4	685·2

aspartate and glutamate were labelled after only 3 min incubation with fumarate-2,3-^{14}C. The insoluble residue was labelled early and continued to increase in ^{14}C over the experiment; after 320 min it contained 32% of the absorbed ^{14}C.

Privet roots sluggishly metabolized fumarate-2,3-^{14}C to $^{14}CO_2$ (Table 3) although large amounts of ^{14}C were found in malate and lesser amounts in citrate, succinate and α-ketoglutarate. Privet roots, in contrast to peach and apple roots, continued to accumulate fumarate-^{14}C suggesting that fumarate may have been located in a cytoplasmic pool. Significant amounts of ^{14}C were incorporated into glutamate and aspartate, and privet roots were similar to peach roots in incorporating a larger amount of ^{14}C into aspartate than glutamate. Privet roots incorporated only 9·9% of the ^{14}C absorbed in 320 min into the insoluble residue.

TABLE 3. THE METABOLISM OF FUMARATE-2,3-^{14}C BY PRIVET ROOTS

Fraction	Min incubated					
	10	20	40	80	160	320
	cpm × 10^{-3}/g fr. wt.					
CO_2	1·6	2·6	1·3	0	0·1	0·7
Amino acids						
Glutamate	0·7	1·2	1·8	4·4	9·1	13·2
Aspartate	2·2	5·1	8·0	10·3	17·5	35·7
All others	0·1	0·5	0·9	2·3	4·1	10·0
Organic acids						
Citrate	0·7	2·4	1·1	4·8	21·3	28·2
Malate	6·1	19·1	19·1	33·3	71·1	178·4
α-Ketoglutarate	0·3	1·4	0	0	0	3·1
Succinate	0·7	1·5	1·6	0·9	3·6	12·5
Fumarate	27·8	23·4	31·4	56·2	81·8	90·8
Sugars	0·2	0·2	0·2	0·3	0·6	1·2
Insoluble residue	0·8	2·2	2·8	5·8	16·8	40·9
Total ^{14}C absorbed	41·2	59·6	68·2	118·3	226·0	414·7

DISCUSSION

In the roots of the three species studied, fumarate, like succinate in corn roots[7] appears to have ready access to the citric acid cycle. The incubation experiments described with fumarate-1,4-^{14}C show the ease by which fumarate-^{14}C was metabolized to CO_2. One turn of the citric acid cycle releases both carbons 1 and 4 of fumarate as CO_2 while carbons 2 and 3 are released on subsequent turns. However, while the bulk of the label from fumarate-1,4-^{14}C was lost as $^{14}CO_2$, little label from fumarate-2,3-^{14}C was metabolized to $^{14}CO_2$ (Fig. 1) suggesting that after one complete turn of the citric acid cycle, the bulk of the organic acids labelled from fumarate were no longer in ready equilibrium with the cycle. This is evidenced by the large pools of malate (Tables 2 and 3), fumarate (Table 3) and citrate (Table 1) found at the end of the experiment. Lips and Beevers[8] have shown that two separate pools of malate exist in corn roots, neither of which appeared to be in the vacuole.

The ^{14}C in the amino acid fraction was primarily in glutamate and aspartate and these amino acids were labelled early and continued to increase in label throughout the experiments. This emphasizes the relationship between these two amino acids and the citric acid cycle.

[7] B. T. STEER and H. BEEVERS, *Plant Physiol.* **42**, 1197 (1967).
[8] S. H. LIPS and H. BEEVERS, *Plant Physiol.* **41**, 709 (1966).

Synthesis of aspartate from oxaloacetate and glutamate from α-ketoglutarate, would eventually decrease the rate at which the cycle could operate unless these losses were offset by a renewal of the supply of these acids. In micro-organisms[9] and plants,[10] the oxaloacetate supply can be replenished by CO_2 fixation. The present studies suggest that the supply of oxaloacetate may also be replenished by cytoplasmic pools of citric acid cycle acids.

The synthesis of amino acids from acids of the citric acid cycle and the rate of their subsequent incorporation into protein (as indicated by the incorporation of label into the insoluble residue) apparently play a role in the sequestering of fumarate carbon into extra-mitochondrial pools. Thus peach roots which synthesized large amounts of protein accumulated only moderate amounts of fumarate carbon in malate and citrate (Table 1). Apple roots synthesized a somewhat smaller amount of protein and synthesized a large pool of malate (Table 2). Privet roots, however, synthesized little protein and large amounts of fumarate carbon were converted into malate or remained unmetabolized. It appears therefore, that peach and apple roots metabolized fumarate as rapidly as it was absorbed, while the slower-growing privet roots, which could not immediately use the absorbed fumarate, shunted this carbon off into extra-mitochondrial pools.

Previous studies[6] with α-ketoglutarate-^{14}C suggested that in woody plants aspartate was formed from fumarate by aspartase or from oxalacetate by oxaloacetic-glutamic transaminase. Glutamate was the only labelled amino acid after 6 min incubation with α-ketoglutarate. The present studies show that fumarate was converted into both glutamate and aspartate in 3 min with the ^{14}C activity predominating in glutamate. If aspartate was formed directly from fumarate (by aspartase), the label should occur in aspartate before glutamate. It appears that synthesis of aspartate by aspartase occurs slowly, if at all, and is probably not physiologically significant in these species.

The labelling data show that although the pathways of metabolism of fumarate may be those expected, the metabolic rates of its utilization in the three species are greatly dissimilar. A comparison of the distribution of label at 320 min by the three species (Tables 1–3) emphasizes this. The amino acid fraction contains approximately the same percentage of absorbed label in all three species. However, the percentage of absorbed label in free amino acids did not approximate to that in protein. Peach roots incorporated 50 per cent of the absorbed label into protein, apple 35 per cent and privet 10 per cent. Similarly, the percentage of absorbed label in organic acids of the citric acid cycle did not approximate to the label found in CO_2. Privet roots contained the most ^{14}C in organic acids but released the least ^{14}C as CO_2. Peach, which incorporated the most label into protein, had the least ^{14}C in organic acids and privet, which accumulated the least ^{14}C in protein, had the most label in organic acids.

In apple trees, glutamate, aspartate, and their amides contain most of the nitrogen found in the xylem tissues.[5] In the present experiments with excised roots, normal translocation through the vascular system was not possible. However, in intact plants some of this newly synthesized glutamate and aspartate could be assumed to be translocated to the aerial tissues as is the case in sugar-beets.[2]

EXPERIMENTAL

Materials and Methods

Plant materials. Peach seeds (*Prunus persica* L. var. Elberta) with the seed coat removed were sterilized by immersion in 0·25% NaOCl for 30 sec. The seeds were rinsed and then cultured on sterile 0·5% agar with

[9] J. M. WIAME, *Advan. Enzymol.* **18**, 241 (1957).
[10] W. E. SPLITTSTOESSER, *Plant Physiol.* **41**, 755 (1966).

nutrient solution. The seedlings were grown for 32 days in a growth chamber under 30,000 lux. Apple roots (*Pyrus malus* L. var. M. Merton 106) were obtained from 1-yr-old rooted cuttings grown for 30 to 37 days in non-sterile medium. Mist-propagated privet (*Ligustrum vulgarea* L.) cuttings were used.

Incubation procedure. Replicate samples (0·5–1 g) of the distal 1 to 2 cm of the roots were placed in No. 15 medium fritted glass filter funnels containing 2·5 μc of fumarate-1,4-^{14}C or fumarate-2,3-^{14}C (30 or 6 μMoles) in 5 ml of 0·5 M potassium phosphate buffer, pH 6·1. Air was pulled through the base of the filter funnels to aerate the roots suspended in solution. Respired CO_2 was carried in the air stream and bubbled through 20 ml of 1 M hyamine hydroxide. The absorbed CO_2 was counted for radioactivity using a liquid scintillation spectrometer.

Analytical methods. At predetermined times an aeration vessel was disconnected from the system, substrate was drained off, and the roots were rinsed with de-ionized water to remove non-absorbed fumarate. The roots were transferred to 50 ml of boiling 100% EtOH and boiled for 10 min. The tissues were then homogenized with a Virtis blender and centrifuged at 10,000 × *g*. The residues were successively extracted four times with 80% (v/v) ethanol. The extracts were combined and evaporated to dryness at 35° in a rotary evaporator.

The soluble extract was then dissolved in water and fractionated sequentially on 1 × 8 cm columns of Dowex 50 (H+) and Dowex 1 (formate) resins.[11] The amino acid fraction eluted from the Dowex 50 (H+) column was separated into an acidic amino acid fraction (glutamate and aspartate) and a neutral and basic amino acid fraction, by passage over a Dowex 1 (acetate) column. This procedure separates the initial extract into acidic amino acid, other amino acid, organic acid, and sugar fractions. The insoluble residue remaining after centrifugation was assayed for radioactivity, hydrolyzed with 6 N HCl for 5 hr in the autoclave at 15 lb/in^2 and then treated in the same manner as the soluble extract. Samples of all of the fractions were assayed for radioactivity.

The components of the organic acid fraction were separated by paper chromatography in n-butanol:90% formic acid:water v/v/v (1:1:1) aged 24 hr.[12] Glutamate and aspartate were separated by paper chromatography using water-saturated phenol.[13] After the chromatograms were dried, the radioactive components were located with a strip scanner.

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[11] W. E. SPLITTSTOESSER, *Plant Cell Physiol.* **10**, 87 (1969).
[12] E. BOGIN and A. WALLACE, *Proc. Am. Soc. Hort. Sci.* **89**, 182 (1966).
[13] K. FINK, R. E. CLINE and R. M. FINK, *Anal. Chem.* **35**, 389 (1963).