METABOLISM OF INFILTRATED ORGANIC ACIDS BY TOBACCO LEAVES[1]

V. ZBINOVSKY AND R. H. BURRIS

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The organic acids are key metabolites in plants, for they function in the synthesis of protein, carbohydrate, and fat and serve as photosynthetic and respiratory intermediates. Their metabolism, which has been receiving increased attention, recently has been reviewed by THIMANN and BONNER (**12**). The present paper describes the conversion of infiltrated C^{14}-labeled formic, oxalic, glycolic, acetic, malic or citric acid to other acids in tobacco leaves.

VICKERY *et al.* (**16, 17**) have excised tobacco leaves, immersed their cut ends in various solutions, and followed the quantitative changes with time in the organic acids of the leaves. The most striking change in the dark was the decrease in malic acid accompanied by an increase in citric acid; all data supported the suggestion that malic acid was the source of the citric acid formed. More recently VICKERY and coworkers (**8, 14, 15**) have immersed the cut ends of tobacco leaves in solutions of the potassium salts of malic, citric, succinic, fumaric, pyruvic, lactic, tartaric, malonic, isocitric, oxalic and acetic acids, and have followed the changes in the dark of total organic acid, oxalic, malic, citric and unknown acids, and acetic and isocitric acids when supplied. The changes observed all appeared compatible with the operation of a tricarboxylic acid cycle in the tobacco leaves.

KROTKOV and BARKER (**6**) allowed a cut tobacco leaf to absorb labeled acetate through the petiole. Much of the acetate disappearing during the 22 hour treatment in the dark was oxidized to carbon dioxide, but part was transformed into an unidentified fraction with the characteristics of an organic acid. STUTZ and BURRIS (**11**) followed the distribution of C^{14} in tobacco leaves supplied with $C^{14}O_2$ in the light and in the dark. In almost all instances, the malic acid had the greatest total and specific activity among the organic acids. Tobacco plants were allowed to fix $C^{14}O_2$ for 15 minutes in the light and then were kept in the dark for four hours in the absence of $C^{14}O_2$. The leaves harvested after 15 minutes in light yielded organic acids with lower C^{14} specific activities than the leaves at the end of the ensuing dark period; evidently high specific activity precursors of the organic acids were formed in the light and transformed to organic acids in the dark.

Materials and methods

Sodium formate labeled with C^{14} was obtained from the Atomic Energy Commission, Isotopes Division, and carboxyl labeled sodium acetate from

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Tracerlab, Inc. The calcium glycolate-2-C^{14} was synthesized by Dr. B. M. Tolbert. Oxalic acid was produced by the controlled heating of labeled sodium formate (**5**). Malic and citric acids were isolated by chromatography on silica gel from the leaves of *Bryophyllum calycinum* which had been exposed to $C^{14}O_2$ in the light.

Tobacco, *Nicotiana tabacum* var. Havana 38, was grown in the greenhouse for one to three months with HOAGLAND and ARNON's nutrient solution number 1 (**3**). Leaves used for infiltration were from 15 to 17 cm. in length and weighed from 3 to 4 gm. Leaves, cut under water, were submerged in a solution of a C^{14}-labeled acid contained in a paraffin-coated dish. The solutions of the calcium or sodium salts of the acids were adjusted to pH 5.5 with H_2SO_4, and one ml. of the solutions gave 5,000 to 20,000 counts per minute when counted with a windowless flowing gas counter. The dish containing the leaf was placed in a vacuum desiccator, evacuated to about 15 mm. Hg residual pressure, allowed to stand for 30 to 60 seconds, and then the vacuum was released slowly. Successful infiltration was achieved only when the leaf blade was uniformly translucent but the main rib and veins showed no indication of infiltration; the leaf then transpired the infiltrated water rapidly but did not wilt when the petiole was immersed in water. Leaves from individual plants were tested to determine the optimum pressure for infiltration. The infiltrated leaves were rinsed twice with distilled water, blotted with paper towels, and placed in a hood. They were uncovered for illumination or were placed in a ventilated cardboard drum for darkening. The leaves transpired their excess water in one to four hours, and they were allowed to metabolize for 30 minutes after they regained their initial appearance. The leaves were then frozen with liquid air or solid CO_2, ground, and extracted for organic acid analysis.

A slurry of the frozen, ground tissue was made in 0.5 *N* H_2SO_4 and 7 *N* H_2SO_4 was added to pH 1, a pH low enough to liberate the free acids. The slurry was mixed with an equal weight of iron-free (HCl washed) celite 545, and 1 : 1 butanol : chloroform, equilibrated with 0.5 *N* H_2SO_4, was added and mixed (100 ml. total solvent per gm. original wet leaf was used for the slurry and for washing). This slurry was placed on a column, and the cylinder of celite was washed with the additional acid-equilibrated butanol-chloroform when the original solvent from the slurry had passed through it. The effluent was caught in 0.1 *N* NaOH (10 ml. per 4 gm. original tissue) to prevent esterification. The acids were titrated, the salts were extracted into water, the solution was concentrated, and the free acids were liberated and mixed with silica gel essentially as described earlier (**4, 11**). In this second purification the free acids were eluted from the silica gel with 1 : 1 tertiary amyl alcohol : chloroform equilibrated with 1 M Na_2SO_4; the total number of ml. of the solvent collected did not exceed the weight in grams of original leaf tissue used.

For the separation of the organic acids a column containing 1 gm. of silica gel was prepared as described earlier (**4, 11**) with about 1.5 gm. of

0.5 N H_2SO_4 (stationary solvent) added to each gm. of silica gel. Mallinckrodt's silicic acid for chromatography can be employed, but the amounts of silica gel and aqueous phase used will differ from those described here (**7**). The acids in the tertiary amyl alcohol : chloroform solvent were added to the column and fractions of the eluting solvent, 35% n-butanol in chloroform mixture equilibrated with 0.5 N H_2SO_4, were collected with an automatic fraction collector. Water, free of carbon dioxide, was added to each tube, and the contents were titrated with 0.05 N $Ba(OH)_2$. Succinic and acetic acids, which are eluted quickly from the chromatographic columns, may be contaminated with other acids, so the tables designate them as the succinic fraction and the acetic fraction.

Samples from the experiments with labeled malic and citric acids were converted to $BaCO_3$ which was distributed on aluminum planchets for counting (**11**). In all other experiments, aliquots of the titrated acids were dried directly on the aluminum planchets. Specific activities were calculated from the amount of carbon present in the individual acids as indicated by their titration. All samples were counted with a windowless flowing gas counter; the counting errors were less than 10%.

Results

A tobacco leaf was infiltrated with C^{14}-labeled formic acid and then was exposed to light for 40 minutes. From the analyses of the organic acids recovered (table I), it is apparent that during the 40 minute period the carbon of formic acid was distributed into a number of other acids. The assimilation of the C^{14} into malic acid was particularly striking, for at the end of the experiment there was far more C^{14} in malic acid than in the formic acid remaining. Not only the total activity but also the specific activity of malic acid was very high. The citric acid in this leaf was abundant, but picked up much less of the C^{14} than did the malic acid. Oxalic acid, glycolic acid, and the succinic fraction each had a higher specific activity than the citric acid. Little glycolic acid is present in tobacco leaves, but it had a high specific activity. Although it may have been formed by the reversal of glycolic and glyoxylic acid dehydrogenases, for the combined activity of these enzymes yields formic acid and carbon dioxide from glycolic acid, the data do not establish that this was the pathway of glycolic acid formation. Degradation of glycolic acid to determine the position of the label derived from formic acid might indicate the pathway of formation of the compound; it is known (**10**) that fixation of $C^{14}O_2$ rapidly yields glycolic acid uniformly labeled.

Formic acid obviously is a very active metabolite in tobacco leaves, for most of it disappeared in 40 minutes and much of its radioactivity appeared in malic acid. Whether this conversion to malic acid involved an initial oxidation to carbon dioxide and a photosynthetic fixation of the CO_2, or whether formic acid itself reacted in malic acid formation cannot be answered by the data, but it seems very likely that a direct conversion without

TABLE I

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED FORMIC ACID AND EXPOSED FOR 40 MINUTES IN THE LIGHT.

Organic acid	Acid	Total counts/min.	Counts / min. × mg. C
	mg.		
Formic	0.53	1,530	11,100
Succinic fraction	0.85	520	1,510
Oxalic	4.16	920	830
Glycolic	0.83	690	2,620
Malic	10.80	25,400	6,570
Citric	14.75	2,360	428

formation of CO_2 was involved. The formic acid remaining had a lower specific activity than that infiltrated, so apparently formic acid is a normal metabolite in the tobacco leaf.

Table II gives the distribution of C^{14} after infiltration of C^{14}-labeled oxalic acid into a tobacco leaf. Oxalate is far from an active metabolite, for after 2.5 hours exposure to oxalate in the dark only a very small percentage of the radioactivity had moved from oxalic acid into the other organic acids. The oxalic acid had a specific activity at the end of the experiment about 40 times as high as that of malic and citric acids, and the succinic fraction acquired virtually no C^{14}. The conclusion of VICKERY *et al.* (**15, 16**) that oxalic acid is an end product and a rather inert compound in the tobacco plant is well verified by these tests with the labeled acid.

Pucher *et al.* (**9**) demonstrated that the concentration of oxalic acid increased rapidly in the buckwheat plant in the early hours of the day. It might be implied that oxalic acid is an active intermediate in the buckwheat plant, although the data also are compatible with the concept that the oxalic acid accumulates as a metabolic end product. Table III shows the distribution of C^{14} among the organic acids of buckwheat leaves infiltrated with

TABLE II

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED OXALIC ACID AND EXPOSED FOR 150 MINUTES IN THE DARK.

Organic acid	Acid	Total counts/min.	Counts / min. × mg. C
	mg.		
Succinic fraction	10.30	23	5
Oxalic	4.86	12,000	9,300
Malic	3.94	314	225
Citric	1.64	163	266

TABLE III

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM BUCKWHEAT LEAVES INFILTRATED WITH C^{14}-LABELED OXALIC ACID AND EXPOSED FOR 40 MINUTES IN THE LIGHT OR 180 MINUTES IN THE DARK.

Organic acid	Acid		Total counts/min		Counts min. × mg. C	
	40 min.	180 min.	40 min.	180 min.	40 min.	180 min.
	mg.	*mg.*				
Volatile acids	3.34	26.20	64	72	47	8
Succinic fraction	0.12	0.62	95	960	1,900	3,830
Oxalic	1.80	15.50	5,630	160,000	11,750	38,800
Glycolic	0.10	0.66	53	1,470	1,700	7,000
Malic	0.92	6.36	377	1,920	1,130	840
Citric	0.31	2.12	60	168	520	210

C^{14}-labeled oxalic acid and kept in the light for 40 minutes or in the dark for three hours. Although appreciable C^{14} passed into succinic, glycolic and malic acids, it is apparent that the total C^{14} and the C^{14} specific activity remained much higher in the oxalic acid than in any other acid. The conversion of oxalic to other acids is several times more rapid than in tobacco and may account for substantial turnover of carbon in buckwheat. The conversion to glycolic acid is noteworthy, for glycolic acid is an active metabolite in leaves and much of the carbon interchanged from oxalic to other acids may pass through it.

Glycolic acid-2-C^{14} was infiltrated into tobacco leaves and these were exposed for 60 minutes in the light (table IV). After 60 minutes the glycolic acid was nearly exhausted, but its specific activity remained higher than the other acids recovered. The total C^{14} and C^{14} specific activity were much higher in malic acid than in citric acid. Oxalic acid had a high specific activity.

When glycolic acid was infiltrated and the leaf was kept in the dark for 180 minutes, the quantitative distribution of C^{14} among the organic acids

TABLE IV

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED GLYCOLIC ACID AND EXPOSED FOR 60 MINUTES IN THE LIGHT.

Organic acid	Acid	Total counts/min.	Counts min. × mg. C
	mg.		
Acetic fraction	0.78	36	115
Succinic fraction	0.24	42	430
Oxalic	0.89	128	540
Glycolic	0.09	33	1,160
Malic	1.28	215	470
Citric	0.35	16	122

TABLE V

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED GLYCOLIC ACID AND EXPOSED FOR 180 MINUTES IN THE DARK.

Organic acid	Acid	Total counts/min.	Counts min. × mg. C
	mg.		
Acetic fraction	0.93	167	450
Succinic fraction	0.40	133	820
Oxalic	1.31	176	500
Glycolic	0.10	42	1,330
Malic	3.99	493	350
Citric	0.43	230	1,420

was distinctly different (table V) from that observed in the light. In the dark as in the light the acetic and succinic acid fractions became radioactive. Oxalic acid was abundant and actually had a specific activity higher than malic acid—it is evident that the conversion of glycolic to oxalic acid occurs both in light and in dark. The most striking result appears in the relative specific activities of malic and citric acids. Whereas in the light, malic acid had a specific activity almost four times higher than citric acid, in the dark citric acid had a specific activity four times higher than malic acid, although it had lower total C^{14}.

In their detailed examination of the metabolism of C^{14}-labeled glycolic acid by Scenedesmus, SCHOU *et al.* (**10**) observed that both malic and citric acids accumulated less than half as much C^{14} in the dark as in the light; in each instance there was about half as much C^{14} in citric as in malic acid. The observations with tobacco are compatible with the suggestion of BENSON and CALVIN (**1**) and CALVIN (**2**) that a tricarboxylic acid cycle, active in the dark, may be suppressed by light. The assumption must be made that glycolic acid, in addition to its oxidation by glycolic and glyoxylic acid dehydrogenases, can participate in a tricarboxylic acid cycle. This assumption is supported by the observation of SCHOU *et al.* (**10**) that over twice as much C^{14} from glycolic acid accumulated in glutamic acid in the dark as in

TABLE VI

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED ACETIC ACID AND EXPOSED FOR 210 MINUTES IN THE DARK.

Organic acid	Acid	Total counts/min.	Counts min. × mg. C
	mg.		
Acetic	0.84	300	895
Oxalic	0.67	4	18
Malic	4.12	830	560
Citric	1.90	690	970

TABLE VII

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED MALIC ACID AND EXPOSED FOR FOUR HOURS IN THE DARK.

Organic acid	Acid	Total counts/min.	Counts min. × mg. C
	mg.		
Succinic fraction	0.48	280	1,440
Malic	1.12	4,170	10,400
Citric	1.13	957	2,260

the light, a fact indicative of high activity of a tricarboxylic acid cycle in the dark and its partial suppression in the light.

When carboxyl labeled acetic acid was infiltrated and the leaf was kept in the dark for 210 minutes (table VI), citric acid had almost twice the specific activity of malic acid, a result much like that observed when glycolic acid was metabolized in the dark. These two-carbon acids may be used in similar fashion in the formation of malic and citric acids. However, as glycolic acid transfers considerable C^{14} to oxalic acid and acetic acid transfers little, it may be assumed that acetic acid yields little glycolic acid. Any substantial reduction of glycolic to acetic acid thus appears highly unlikely. The higher specific activity of citric acid compared with acetic acid indicates that acetic acid is a normal metabolite of tobacco leaves, for only newly produced acetic acid could dilute the radioactivity of the acetic acid infiltrated to a specific activity lower than that of citric acid. In the experiments of KROTKOV and BARKER (**6**) the dilution of the acetate was insufficient to establish this point.

PUCHER, VICKERY *et al.* (**8, 16**) have presented much evidence indicating that, in the dark, malic acid is converted to citric acid in tobacco leaves. The isotopic technique offers an independent means of checking this observation. Malic acid labeled with C^{14} was infiltrated into a tobacco leaf and the leaf was kept in the dark for four hours. Table VII indicates the spe-

TABLE VIII

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM TOBACCO LEAVES INFILTRATED WITH C^{14}-LABELED CITRIC ACID AND EXPOSED FOR FOUR HOURS IN THE DARK.

Organic acid	Acid	Total counts/min.	Counts min. × mg. C
	mg.		
Succinic fraction	1.99	63.2	78
Oxalic	0.45	5.6	47
Glycolic	0.24	0.4	6
Malic	4.25	15.5	10
Citric	2.36	1,645.0	1,850

cific and total activities of the acids. As malic acid was infiltrated it had the highest specific activity, but citric acid also had a high specific activity. These data leave no question that carbon from malic acid can be used in the synthesis of citric acid in the darkened tobacco leaf. The small amount of succinic acid formed could have arisen by direct reduction of malic acid, but the higher specific activity in citric acid compared with succinic acid is compatible with the alternative that the succinic acid was formed by way of the tricarboxylic acid cycle.

The specific activities of acids isolated from leaves infiltrated with C^{14}-labeled citric acid and kept four hours in the dark are given in table VIII. The citric acid was converted to other acids sluggishly when compared with acids such as formic, acetic and malic. After four hours the citric acid had 24 times as high a specific activity as the next most active fraction. Malic acid was formed slowly from the citric acid. Less activity appeared in glycolic than in oxalic acid in four hours.

Discussion

The data presented illustrate clearly the rapid interconversion of the organic acids in the tobacco leaf. However, there are certain limitations to the experiments that should be considered. First, the tests have been made at pH 5.5 only, and penetration of the labeled compounds into the cells may be better at a lower pH. Second, although incorporation of C^{14} via CO_2, arising from oxidation of the labeled acids, is probably of minor importance, such incorporation is not distinguished from incorporation directly of intact carbon chains. Third, compounds other than the organic acids separated unquestionably assimilate a substantial fraction of the C^{14} from the infiltrated acid. Fourth, lack of replication, high sampling error and variation in time of exposure preclude assertion that the values reported are absolute, although the data are adequate to show the general magnitude of the interconversions of the organic acids in detached tobacco leaves.

With the above limitations defined, an examination of the data indicates a number of clear-cut interconversions of organic acids by the tobacco leaf. One of the most striking is the rapid conversion of formic to malic acid. The data in addition to revealing the rapid utilization of formic acid indicate that it is a normal metabolite in the tobacco leaf. It may arise from the oxidation of glycolic and glyoxylic acids (**13**).

The sluggish response of the leaves to infiltrated oxalic acid is not surprising, for most previous work has indicated it to be an end product of metabolism. The problem of permeability may be a particularly pertinent one here, for the pK_a of oxalic acid is well below that of the other acids used. Assuming that oxalic acid penetrated effectively, there is nothing to indicate it is an active compound in tobacco; however, in buckwheat it may be an important intermediate in acid metabolism.

Malic acid, citric acid and acetic acid (as an active derivative) participate in the tricarboxylic acid cycle. Much evidence suggests the existence

of a tricarboxylic acid respiratory cycle in plant tissues, although detailed support for its function in any single plant tissue has not been marshalled. Determination of the fate of infiltrated labeled acids may reveal the functioning of a cyclic respiratory mechanism. Acetic acid quickly transferred C^{14} to malic and citric acids. If the citric acid arose by condensation of active acetate with oxalacetic acid and then gave rise to malic acid through the usual tricarboxylic acid cycle, both the malic and citric acid would be labeled with C^{14}. The high specific activity of the citric acid fits the concept well that it was formed from active acetate by the condensing enzyme, and the lower radioactivity of malic acid is compatible with its production from the citric acid.

When labeled malic acid was infiltrated into tobacco leaves kept in the dark, citric acid acquired C^{14}, a result one would predict if a tricarboxylic acid cycle were operative. The fact that the succinic acid fraction recovered had a lower specific activity than the citric acid is compatible with its formation from citric acid in the cyclic oxidation process, though it does not prove that it did not arise from reduction of malic via fumaric acid. When the results from the infiltration of labeled citric acid are compared with those from the infiltration of malic acid, it is apparent that in four hours in the dark the succinic fraction had a relatively higher specific activity when derived from malic than from citric acid. Although caution must be used in interpreting the data in absolute terms, these results raise some questions regarding the activity of the cycle. The very low C^{14} content in malic acid four hours after labeled citric acid was infiltrated is indicative of a slow operation of a tricarboxylic acid cycle at best, although permeability difficulties may be evoked in explanation. In short, the data are compatible with the operation of a tricarboxylic acid cycle in detached tobacco leaves but do not indicate that it functions rapidly; its part in the overall respiration of the plant remains to be established.

Summary

Detached leaves of tobacco were vacuum infiltrated with solutions of C^{14}-labeled formic, oxalic, glycolic, acetic, malic or citric acids adjusted to pH 5.5. After the leaves metabolized in the light or dark, their organic acids were recovered and were analyzed for C^{14}.

Formic acid apparently is a normal metabolite in tobacco leaves. It was converted rapidly to other organic acids and particularly high levels of C^{14} appeared in malic acid.

Oxalic acid was rather inert in tobacco but was more active in buckwheat leaves.

When labeled glycolic acid was metabolized in the dark, the ratio of C^{14} in citric/malic acid was markedly higher than in the light. Both in light and dark much C^{14} from glycolic acid accumulated in oxalic acid.

In the dark, acetic acid yielded citric acid of about twice the specific activity of malic acid. Acetic acid did not appear to be oxidized by way of glycolic acid.

Malic acid was converted to citric acid by the darkened tobacco leaf.

Citric acid was converted sluggishly to other organic acids.

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF WISCONSIN
MADISON 6, WISCONSIN

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