Compartmentation of Organic Acids in Corn Roots[1]
I. Differential Labeling of 2 Malate Pools

S. Herman Lips[2] and Harry Beevers
Department of Biological Sciences, Purdue University, Lafayette, Indiana

Summary. Bicarbonate-^{14}C and acetate-^{3}H were simultaneously provided to corn roots to give 2 isotopic forms of malate in the tissue, malate-^{14}C produced by dark fixation reactions and malate-^{3}H produced by reactions of the tricarboxylic acid cycle. Following a short pulse of exposure to the isotopes, the dissimilation of both isotopic forms of malic acid was followed. The rate of utilization of malate-^{3}H was much faster than that of malate-[14]C.

These results are interpreted as showing that the malate produced from [14]CO_2 is in a pool physically separated from that in the tricarboxylic acid cycle. The introduction of the 2 isotopes through distinct metabolic pathways produced the differential labeling of 2 distinct pools of malate.

In previous experiments from this laboratory it has been shown, by measuring specific activities of CO_2 and the carboxyl groups of some organic and amino acids after providing acetate-1-^{14}C, that the amounts of the individual acids undergoing metabolic turnover in the tricarboxylic acid cycle in a variety of plant tissues may be only a small fraction of the total amount of acid present; in a word, that compartmentation of these metabolites is a general feature of plant cells (3). The present experiments were designed to characterize further the pools of organic acid and to determine their response to different experimental and physiological conditions.

The establishment of separate pools of a particular compound within the cells can presumably be achieved by 2 mechanisms: A) the compartmentized metabolite has a single origin and is segregated into distinct pools after its synthesis (e.g. an organic acid is produced by the tricarboxylic acid cycle within the mitochondria but part of it is excreted into the extramitochondrial cytoplasm or into the vacuole), and B) the pools have different origins, are specifically connected to distinct metabolic pathways and are not in equilibrium. These mechanisms are not exclusive; they may operate simultaneously and intermediate situations are possible.

As a first step we concentrated on the second mechanism (B) for which purpose an organic acid that could be synthesized by 2 different pathways at sufficiently high rates was necessary. Malic acid was found to be especially well suited for this purpose. In this paper, then, we concentrate on the malate component of subapical segments of primary corn roots, of which a considerable fraction (more than 60 %) was shown not to be in equilibrium with the turnover pool in the tricarboxylic acid cycle (3). In this tissue acetate is rapidly metabolized, almost exclusively, by the tricarboxylic acid cycle (2). Having established that the malate could be heavily labeled by supplying either acetate-[3]H (tricarboxylic acid cycle malate) or bicarbonate-[14]C (dark fixation malate) we performed double labeling experiments in which both isotopes were supplied for a brief period (the pulse) and followed the kinetic behavior of total malate and each of the labeled forms during a subsequent period of 3 hours. From the distinctly different behavior of the 2 labeled forms of malate it was deduced that they are in separate pools, and a further degree of compartmentation of this metabolite is thus established.

Materials and Methods

Maize grains (var. Wf 9 × 38-11) obtained from the Agricultural Alumni Seed Association, Lafayette, Indiana, were soaked in tap water for about 17 hours and germinated for 2 days at 30° between moist paper towels. The root tips (apical 5 mm) were removed and subapical segments (2 cm) were used as experimental material.

The usual procedure was to place 2.5 g samples of root segments in open petri dishes containing 20 ml 0.01 M potassium phosphate pH 7.5 and the labeled acetate and/or bicarbonate. The amounts of labeled material used in the various experiments were as follows: Na-acetate-[3]H: 50 μc (0.1 μmole), Na-acetate-1-[14]C: 5 μc (17.5 μmoles), and Na-bicarbonate-[14]C: 10 μc (0.55 μmole).

After 15 minutes the incubation medium was rapidly removed by suction. The roots were washed with about 50 ml water and the rinse water was immediately removed. The incubation after the 15-minute pulse was carried out in fritted glass funnels in 20 ml 0.01 M potassium phosphate, at the stated pH, through which a vigorous moist air stream was passed. At intervals the sample of roots from a single funnel was removed, transferred to 30 ml ethanol, and ground in a Virtis 45 homogenizer for 1 to 2 minutes. The homogenate was then filtered under suction and the residue extracted wtih ethanol. The combined extracts were dried in a rotary evaporator at 50° and the lipids removed with chloroform. The lipid-free extract was dissolved in water and fractionated on columns of Dowex resin according to Canvin and Beevers (1).

The organic acids in the anion fraction were separated on a GME fraction collector using a linear gradient of formic acid from zero to 4 N. The test tubes containing the fractions were dried and 1 ml water added to each; after titration with standard NaOH, samples of 0.5 ml were taken for radioactivity determination in a 3 channel Packard scintillation spectrometer.

The scintillation fluid consisted of: 350 ml toluene, 350 ml dioxane, 210 ml methanol, 73 g naphthalene, 4.52 g PPO, (2,5-diphenyloxazole), and 0.078 g POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene]. Samples were counted in polyethylene vials with an efficiency of 14 % for ^{3}H and 23 % for ^{14}C in double label determinations. Using discriminator settings of 30 % gain, 50 to 150 for the first channel and 7.5 % gain, 150 to 1000 for the second channel, no ^{3}H was detected in the second channel and about 20 % of the carbon appeared in the first channel. Calculations of counting efficiency of ^{3}H and ^{14}C were done by the internal standard method and by quenching samples of ^{3}H-toluene and ^{14}C-toluene with 0.5 ml of water as present in the samples of organic acids counted in the experiments.

Results

After a 15-minute pulse in acetate-1-^{14}C at pH 5.0 the ^{14}C in malate accounted for 15 to 20 % of that in the organic acid fraction. A similar pulse of ^{14}C labeled bicarbonate at pH 7.5 gave rise to malate-^{14}C which contained 96 % of the ^{14}C in the organic acid fraction.

During the subsequent incubation at pH 7.5 the malate-^{14}C produced from acetate-1-^{14}C behaved as if it were all in a turnover pool of the tricarboxylic acid cycle which was continuously replenished by endogenous unlabeled acetate (fig 1A). After an initial rise in the ^{14}C content which would be expected if it were produced from labeled precursors remaining at the end of the pulse, malate-^{14}C declined steadily to a very low value by 3 hours. (In fig 1A and subsequent figures the curves show isotopic content
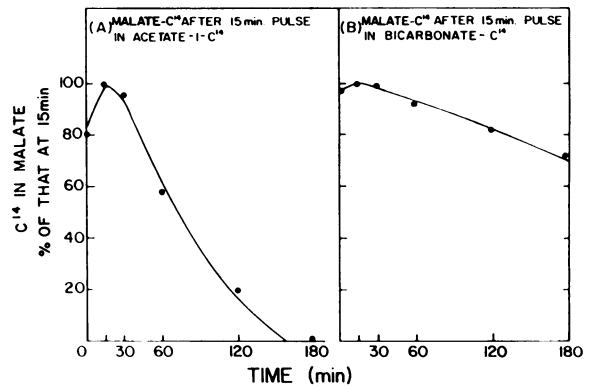

FIG. 1. Utilization of malate-^{14}C at pH 7.5 following a 15-minute pulse, (A) in acetate-1-^{14}C and (B) in bicarbonate-[14]C.

of the named component as percent of that present 15 minutes after the pulse.)

The behavior of the labeled malate produced from bicarbonate-^{14}C was quite different. Only a relatively small fraction was metabolized in 3 hours (fig 1B).

The contrasting behavior of malate-^{14}C in these 2 experiments would clearly not have been observed if the malate produced from each of the precursors shared a common pool, and suggested at once that the malate produced by dark fixation of CO_2 was physically separate from that produced in the tricarboxylic acid cycle from acetate, which was actively metabolized.

However, in these experiments, although other conditions were strictly similar, the pH conditions and the exogenous substrates during the pulse were different, and alternative explanations for the different behavior of the resulting malate-^{14}C, though unlikely, could not be ruled out.

This was an additional reason for performing double label experiments in which the 2 types of relevant reactions could be followed simultaneously and
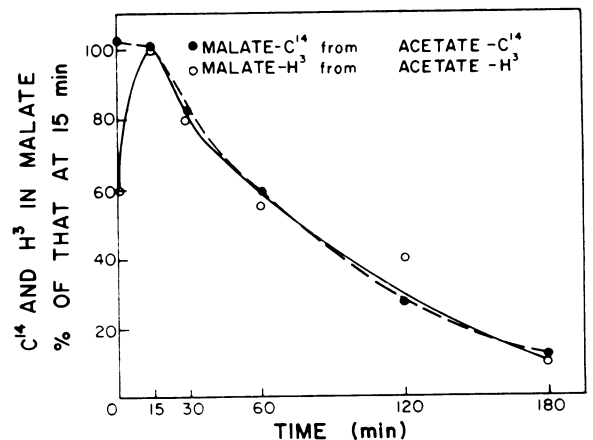

FIG. 2. Utilization of labeled malate at pH 5.0 following a 15-minute pulse in acetate-1-^{14}C plus acetate-[3]H.

under identical conditions. In these, tritium labeled acetate was used to label the malate in the tricarboxylic acid cycle. Because of possible complications of exchange reactions between ^3H and H atoms the validity of regarding ^3H loss from malate following a pulse of acetate-^3H as a measure of malate utilization was first established. This was done by comparing the behavior of labeled malate when it was produced from either acetate-1-^{14}C or acetate-^3H in the same experiment. As figure 2 shows, the disappearance of ^3H and ^{14}C from malate after the pulse followed very similar kinetics. In subsequent experiments acetate-^3H and bicarbonate-^{14}C were present together during the pulse at pH 7.5. Even at this relatively high pH the uptake of acetate (^3H) by the tissue was active enough to label several organic acids and glutamate and aspartate with several thousand counts (table I).

The results of 1 such experiment in which the pH after the pulse was 7.5, are shown in figure 3. It is clear that whereas the malate in the tricarboxylic acid cycle (now labeled with ^3H) was lost quite rapidly, as in the experiment of figure 1A, there was virtually no utilization of ^{14}C labeled (dark fixation) malate.
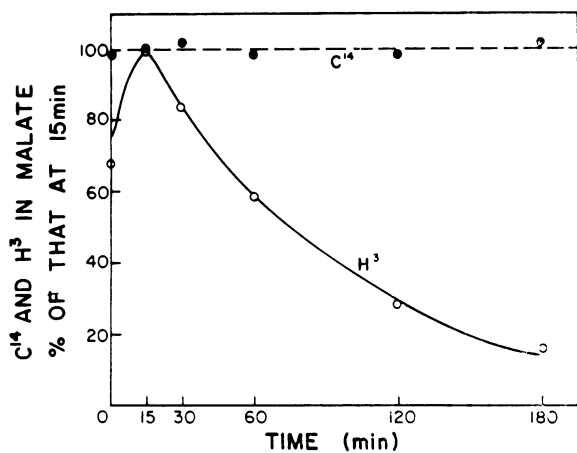
An interesting contrast to the behavior of malate is shown by that of citrate in the double label experiments. As shown in figure 4 the kinetics of ^{14}C and ^3H disappearance from citrate are very similar. This shows that the portion of fixed CO_2 which appears in citrate labels the same citrate pool (presumably the turnover pool in the tricarboxylic acid cycle) as that labeled by acetate-^3H. The possibility of the coexistence of different pools of citrate is not negated by these results which point out only that under the experimental conditions described (short pulse) both isotopic forms of citrate are in the turnover pool and are therefore diluted and oxidized by the metabolic events of the cell to the same extent and at the same rate.
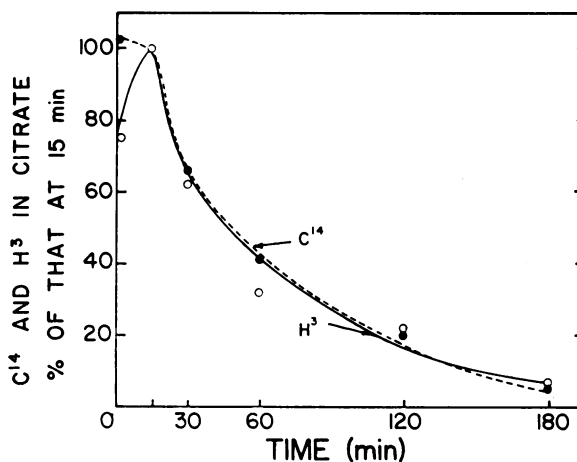

FIG. 4. Utilization of labeled citrate at pH 5.0 following a 15-minute pulse in acetate-^3H plus bicarbonate-^{14}C.

Discussion

The present observations show that for each metabolic pathway considered, the tricarboxylic acid cycle and dark CO_2 fixation, there is a specific pool of malic acid. When the tissue was maintained at pH 7.5 the malate produced from $^{14}CO_2$ was apparently isolated from the oxidative reactions which degrade malate in the cycle.

FIG. 3. Utilization of labeled malate at pH 7.5, following a 15-minute pulse in acetate-^3H plus bicarbonate-^{14}C.

Table I. *Progress of Utilization of Several Compounds at pH 5.0 following 15-Minute Pulse in Acetate-^3H plus Bicarbonate-^{14}C*

The values for ^3H and ^{14}C are in thousands of dpm.

Time (Min)	Succinate ^3H	Succinate ^{14}C	Malate ^3H	Malate ^{14}C	Citrate ^3H	Citrate ^{14}C	Glutamate ^3H	Glutamate ^{14}C	Aspartate ^3H	Aspartate ^{14}C
0	10.0	...	4.5	162.6	3.4	9.5	18.8	16.5	31.2	124.1
15	25.1	...	13.2	150.2	9.4	10.0	46.5	57.6	14.9	58.7
30	7.9	0.2	6.4	136.8	2.9	9.2	26.2	57.5	10.3	33.6
60	7.8	0.1	7.0	129.6	2.0	3.8	16.8	45.8	7.1	23.0
120	5.2	0.1	5.8	108.2	1.4	2.0	9.7	26.6	4.3	9.8
180	3.9	...	2.2	99.2	0	0	9.3	23.9	2.4	7.5
μmoles	2.90		31.3		1.80		6.7		4.3	

The rates of oxidation of the ^{3}H and ^{14}C labeled forms of succinate, citrate, aspartate, and glutamate after the acetate plus bicarbonate pulse are similar (table I). In general it seems that they are labeled with ^{3}H and ^{14}C only through reactions of the tricarboxylic acid cycle, labeling exclusively turnover pools. Pools of these acids other than those related to the cycle are apparently not labeled under the present experimental conditions (short pulse).

Only limited deductions about the intracellular location of the different malate pools may be made from these results. We assume that the malate pool of the tricarboxylic acid cycle is in the mitochondria and thus that the pool of malate produced by CO_2 fixation is outside these organelles. However it does not follow that the malate-^{14}C is in the vacuole, and the bulk of the intracellular malate may be segregated in yet a third distinct compartment.

Literature Cited

1. CANVIN, D. T. AND H. BEEVERS. 1961. Sucrose synthesis from acetate in the germinating castor bean: Kinetics and pathway. J. Biol. Chem. 236: 988–95.

2. HARLEY, J. L. AND H. BEEVERS. 1963. Acetate utilization by maize roots. Plant Physiol. 38: 117–23.

3. MacLENNAN, D. H., H. BEEVERS, AND J. L. HARLEY. 1963. Compartmentation of acids in plant tissues. Biochem. J. 89: 316–27.