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Dark Fixation of CO_2 by Crassulacean Plants

EVIDENCE FOR A SINGLE CARBOXYLATION STEP

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ABSTRACT

Malic acid isolated from *Bryophyllum pinnatum* (Lamk.) Oken (*B. calycinum* Salisb.), *Bryophyllum tubiflorum* Harv., *Kalanchoë diagremontiana* Hamet *et* Perrier and *Sedum guatamalense* Hemsl. after dark $^{14}CO_2$ fixation was degraded by an *in vitro* NADP-malic enzyme technique. In the short term (5 to 30 seconds) the malic acid was almost exclusively labeled in the C-4 carboxyl carbon (greater than 90%). The percentage of ^{14}C in the C-4 carboxyl of malic acid declined slowly with time, reaching 70% in *B. tubiflorum* and 54% in *B. pinnatum* after 14 hours of exposure to $^{14}CO_2$. It was found that malic acid-adapted *Lactobacillus arabinosus* may seriously underestimate the C-4 carboxyl component of label in malic acid-^{14}C. The amount of substrate which the bacteria can completely metabolize was easily exceeded; there was a significant level of randomization of label even when β-decarboxylation proceeded to completion, and in extended incubation periods, more than 25% of label was removed from malic acid-U-^{14}C. The significance of these findings in relation to pathways of carbohydrate metabolism and malic acid synthesis in Crassulacean acid metabolism is discussed.

Carbohydrate metabolism in the dark in green tissues of succulent plants, which results in a quantitative inverse relationship between starch content and malic acid level (11, 27), poses the question of regulated carbon flow through the glycolytic and pentose phosphate pathways. As currently envisaged, the P-enolpyruvate consumed in dark CO_2 fixation is derived from intermediates of the pentose phosphate pathway, rather than from glycolysis (25). The evidence for these events hinges on the observed distribution of ^{14}C within malic acid-^{14}C isolated from tissues showing CAM[2]. After short and long term $^{14}CO_2$ fixation in the dark, Bradbeer *et al.* (6) reported that the distribution of label between the C-4 and C-1 carboxyl carbons of malic acid approximated to a constant ratio of 2:1. Varner and Burrell (26) had previously reported an asymmetric distribution of label between the carboxyls of malic acid in *Bryophyllum calycinum* and the approximate 2:1 labeling pattern has been described in other succulent tissues in several subsequent reports (3, 9). Bradbeer *et al.* (6) interpreted this labeling pattern in terms of two carboxylation events, the first via ribulose diphosphate carboxylase and the second via P-enolpyruvate carboxylase, as shown in Figure 1. Very little further support for this hypothesis has been advanced (21, 23).

Labeling patterns in malic acid formed following preillumination and in dark fixation malic acid which approach 2:1 have been reported in other tissues (14, 22). In all experiments, except those of Varner and Burrell (26), the labeling of the β-carboxyl of malic acid was determined by means of malic acid-adapted *Lactobacillus arabinosus*. This technique was developed from the observations of Blanchard *et al.* (4) and was originally used as a manometric assay for malic acid by Nossal (16, 17). The application of this technique to the degradation of labeled malic acid demands that the only significant fate of labeled malic acid fed to the organism is complete β-decarboxylation via the endogenous NAD-malic enzyme. The results presented here demonstrate that this is not always true. Using a more satisfactory *in vitro* degradation procedure, it is shown that labeled malic acid isolated from succulent tissues after dark $^{14}CO_2$ fixation does not show a constant 2:1 labeling pattern. A preliminary report of these findings has been presented elsewhere (24).

MATERIALS AND METHODS

Growth of Plant Material. Stocks of *Bryophyllum pinnatum* (Lamk.) Oken (*B. calycinum* Salisb.), *Bryophyllum tubiflorum* Harv., *Kalanchoë daigremontiana* Hamet *et* Perrier, and *Sedum guatamalense* Hemsl. were maintained in the glasshouse by vegetative propagation. The plants were grown in a sandy clay, adequately watered. Experimental plants were kept in a controlled environment chamber, 12 hr (25 C) light and 12 hr (17 C) dark. The humidity was kept constant at 65% relative humidity.

Plants were used when about 3 months old. The phyllodes in the sixth to eighth whorl from the top of *B. tubiflorum*, the third pair of opposite leaves of *K. daigremontiana*, the second pair of opposite leaves of *B. pinnatum* and the top six whorls of *S. guatamalense* phyllodes were used in experiments.

Estimation of Malic Acid Content of Crassulacean Tissue. A weighed amount of phyllode tissue was killed in boiling water, homogenized with an Ultra-Turrax homogenizer (Janke and Kunkel, Cologne), and exhaustively extracted with boiling water. The homogenate was centrifuged, and the supernatant was decanted.

Malic acid was determined by the enzymatic method of Hohorst (8) with the following modifications. A control sample containing all the reaction components except malic acid was included and absorbance measurements were taken only at the completion of the reaction. The reaction was conducted in 5-ml test tubes in a waterbath at 28 C. The amount of malic acid present was determined by reference to a standard curve

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[2] Abbreviation: CAM: Crassulacean acid metabolism.

of absorbance against malic acid concentration and was expressed as μequivalents per gram fresh weight of tissue.

Dark Fixation of $^{14}CO_2$. Tissue from all four species was exposed to $^{14}CO_2$ during the period of most active acid synthesis (3–6 hr after darkening). $^{14}CO_2$ was generated in an evacuated 10-ml vial by the addition of 20% lactic acid to $NaH^{14}CO_3$ (60 mc per mmole). The acidified reaction was heated in a beaker of boiling water, and the $^{14}CO_2$ was withdrawn with a 10-ml hypodermic syringe.

For long term exposures (5 min and 2, 5.5, and 14 hr) about 1 g of plant material was exposed to 10 to 25 μc of $^{14}CO_2$ in a closed 150-ml or 2.5-liter plastic vessel equipped with a rubber seal through which the $^{14}CO_2$ was injected. The phyllodes were supported in the vessel with the petiole in water. At the end of the exposure period, the tissue was quickly removed and killed in boiling 80% ethanol. For short term exposures (5, 10, 30, and 60 sec), about 4 g of plant material were exposed to 50 μc of $^{14}CO_2$ in a 75-ml chamber fitted with a timer-actuated trapdoor. At the end of the exposure period, the phyllodes were automatically released into a beaker of boiling 80% ethanol, and the residual $^{14}CO_2$ was drawn out of the chamber. During the 5.5- and 14-hr exposure to $^{14}CO_2$, vessels were kept in an 18 C cabinet; all other experiments were done at room temperature. The tissue was killed, sliced, and extracted in 80% ethanol, 50% ethanol, and water. The extracts were pooled.

Isolation of Malic Acid. Malic acid was isolated in two different ways. In the first method, pooled extracts were washed through 2-cm × 4-mm columns of Dowex-1 formate (200–400 mesh) resin with 10 bed volumes of water to remove basic and neutral compounds. The acidic components were eluted from the column with 10 bed volumes of 10 N formic acid followed by two bed volumes of 0.5 N HCl. The acid fraction was evaporated to dryness in a Buchi Rotavapor and taken up in 0.5 ml H_2O. An aliquot of this was then chromatographed on Whatman No. 3MM paper in the organic phase of *tert*-amyl alcohol-formic acid-water (3:1:3, v/v). Radioactivity was localized by autoradiography, and malic acid was identified by comparison with authentic malic acid-^{14}C. The malic acid was eluted from the chromatogram and purity was established by two-dimensional chromatography on Whatman No. 4 paper in ethanol-NH_3-H_2O (4:1:1, v/v) in the first dimension and diethyl ether-formic acid-H_2O (7:2:1, v/v) in the second, followed by autoradiography.

The second method for isolation of malic acid was used for extracts of tissue after short exposure to $^{14}CO_2$. The initial pooled extract was dried in a Buchi Rotavapor and taken up in 1 ml of H_2O. Lipids were extracted, and proteins were denatured by extraction with chloroform (4:1 by volume). Malic acid was then isolated from the lipid-free extract by gradient elution chromatography on a 5-ml column of Dowex-1 formate (200–400 mesh) resin with an increasing formic acid gradient (19) and was shown to contain no radiochemical contaminant when cochromatographed with malic acid.

Preparation of *Lactobacillus arabinosus* Suspensions. The medium used in all phases of culture was modified after that of Nossal (16) and consisted of 20 g of glucose, 20 g of DL-malate, 10 g of Difco yeast extract, 10 g of Difco meat extract, 10 g of sodium acetate trihydrate, 1 g of K_2HPO_4, 10 mg of NaCl, 200 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 4H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$ in 1 liter. The initial pH was 5.5.

The bacteria were obtained from the Australian Wine Research Institute, Adelaide and were maintained on agar slants at 4 C. Each slant provided innoculum for a 10-ml subculture, which was, in turn, used to innoculate a 1 liter bulk culture. All subcultures and bulk cultures were incubated at 28 C for 24 hr. The cells were harvested by centrifugation, washed thoroughly with distilled water, and finally suspended in a minimum volume of 0.1 M KCl, 5 mM $MnCl_2$. This suspension was stored at 4 C.

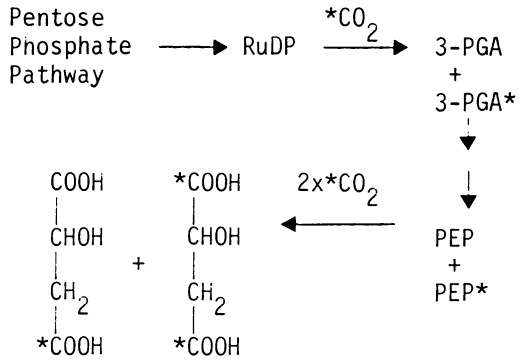

FIG. 1. The double carboxylation scheme for dark $^{14}CO_2$ fixation in CAM (6). RuDP: ribulose diphosphate; PEP: P-enolpyruvate; 3-PGA: 3-phosphoglycerate.

DEGRADATION PROCEDURES

Malic Acid Degradation by Malic Enzyme *in Vitro*. Malic acid was degraded *in vitro* by malic enzyme isolated from maize leaves as described by Hatch (7), with the following modifications. A 52 to 65% ammonium sulfate fraction of a maize leaf extract prepared in 50 mM glycylglycine, pH 7.5, 10 mM 2-mercaptoethanol and 2 mM $MnCl_2$, was used as a source of malic enzyme. No fumarase activity could be detected using the method of Racker (20). The protein was stored as a suspension in 100% ammonium sulfate at 4 C. When required, an aliquot was centrifuged, and the protein pellet was dissolved in the required volume of 75 mM tris-HCl, pH 8.0. Malic enzyme activity was assayed as described by Johnson and Hatch (10). In all degradations using NADP-malic enzyme, the malic acid concentration of the sample to be degraded was first determined using the Hohorst assay (8). This proved to be essential for samples from malic acid rich plants to prevent substrate overloading of the assay and consequent incomplete degradation.

The reaction mixture for malic acid degradation consisted of 0.2 to 0.5 μmole labeled malic acid, 2.5 μmoles of NADP, 1.0 μmole of $MnCl_2$, 7.5 μmoles of tris-HCl, pH 8.0, 2 μmoles of hydrazine dihydrochloride, pH 8.0, and 0.1 unit of malic enzyme. The pyruvate produced was trapped as the hydrazone, because commercial preparations of glutamic-pyruvic transaminase used to drive the reaction towards pyruvate (7) contained unacceptable levels of NADP glutamic dehydrogenase which depleted the NADP available to malic enzyme. The reaction mixture was incubated at 30 C for 30 to 60 min, after which an aliquot (0.1 ml) was spotted onto a 2.5 cm Whatman GF/A glass fiber filter disc, dried in air to remove $^{14}CO_2$, and counted in 3 ml of a toluene scintillation fluid (5 g of PPO in 1000 ml toluene). For each sample, two reaction mixtures, one with and one without enzyme (control), were prepared and treated identically. Control assays with malic acid-U-^{14}C and malic acid-4-^{14}C were always run with the unknown and yielded 24.6 ± 0.7% and 96.7 ± 0.5% of ^{14}C in the C-4 position, respectively.

Degradation of Radioactive Malic Acid by *L. arabinosus*. The reaction medium, unless otherwise specified, consisted of 0.1 ml of 0.3 M sodium acetate buffer, pH 5.0, 0.02 ml of radioactive malic acid sample diluted with 100 mM carrier malic acid (2 μmoles), and 0.5 ml of KCl suspension medium containing 10 mg dry weight of bacteria. A control sample of the

Table I. *Decarboxylation of Malic Acid-4-^{14}C by L. arabinosus under Anaerobic and Aerobic Conditions in Sealed Flasks*

Standard assays were run under N_2 or air and stopped at the indicated time by the addition of trichloracetic acid. Values shown are cpm collected as $^{14}CO_2$ in Hyamine (C) and cpm loss from the reaction mixture (L) at the indicated elapsed time. Each reaction mixture initially contained 66 × 10^3 cpm.

Elapsed Reaction Time	Conditions of Reaction			
	Anaerobic		Aerobic	
	C	L	C	L
min	*cpm* × 10^3			
2	8.1	8.4	12.5	14.1
10	48.1	48.2	51.1	48.9
20	53.4	50.1	53.4	51.4
60	43.9	55.9	56.7	56.5
240	55.4	58.1	53.6	57.9

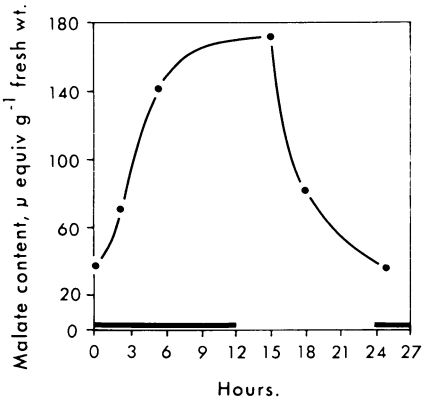

FIG. 2. Diurnal fluctuation of malic acid content of phyllodes from *B. tubiflorum* maintained in a controlled environment.

same composition but without bacteria was used. Both control and bacterial samples were incubated at 30 C. At required times, a 0.1-ml aliquot of each was removed to a scintillation vial containing 0.1 ml 10 N formic acid and heated gently to stop the reaction and drive off evolved CO_2. Five ml of dioxane scintillation fluid (100 g of naphthalene and 5 g of PPO in 1000 ml of dioxane) were added, and the sample was counted. Good agreement between loss of ^{14}C from the reaction mixture and collection of ^{14}C as $^{14}CO_2$ was regularly obtained (Table I) and anaerobiosis (23) had no effect on the assay.

Malic acid-U-^{14}C and NaH $^{14}CO_3$ were obtained from the radiochemical center, Amersham, U.K. Malic acid-4-^{14}C was provided by Dr. M. D. Hatch and was prepared as described (7). Commercially available malic acid-4-^{14}C (Calbiochem) contained significant radioactivity outside the C-4 carboxyl carbon.

RESULTS

Degradation of Malic Acid Formed during CAM. Figure 2 shows the diurnal fluctuation in malic acid content of phyllodes of *B. tubiflorum*, maintained as described previously. The time course of acidification was similar in the other species used, but the extent of acidification varied between species.

Material for dark fixation experiments was collected during the phase of most rapid acid accumulation (3–6 hr after darkening) and was exposed to $^{14}CO_2$ in the dark for periods ranging from 5 sec to 14 hr. Malic acid was the principal labeled product of dark $^{14}CO_2$ fixation in all experiments. For example, the distribution of label after 5-min dark $^{14}CO_2$ fixation was malic acid 78%, aspartic acid 16%, succinic acid 3%, and other compounds 3%. After 2 hr dark $^{14}CO_2$ fixation the distribution was malic acid 70%, citric acid 11%, succinic acid 14%, and aspartic acid 0.75%.

Labeled malic acid was isolated from leaf extracts and degraded by the NADP-malic enzyme technique. The results of these experiments show that in the shortest times (5, 10 sec) malic acid was predominantly labeled in the β-carboxyl (C-4) position during dark fixation in four species of plants showing Crassulacean acid metabolism (Fig. 3). As the time of exposure increased, so did the proportion of label remaining in the pyruvate residue of the assay which corresponds to the C-1 to C-3 moiety of malic acid. This residue was not further degraded. After 14 hr exposure to $^{14}CO_2$ in the dark, only 54% of the label in malic acid from *B. pinnatum* leaves remained in the C-4 position. However, the movement of label from the C-4 position in malic acid of *B. tubiflorum* leaves ceased after 5.5 hr, with 70% of the label remaining in that position.

In another experiment, *B. tubiflorum* phyllodes were exposed to $^{14}CO_2$ for 5 min in the dark or light at different stages of the diurnal cycle. The tissue was equilibrated in dark or light for 10 min prior to exposure to $^{14}CO_2$. Malic acid only contained significant amounts of ^{14}C when tissue acid content was low (3, 12). However, at all stages, dark fixation malic acid was predominantly C-4 labeled (Table II). Malic acid labeled in the light showed a lower proportion of ^{14}C in the β-carboxyl position.

Degradation of Malic Acid by *L. arabinosus*. The degradation of malic acid-U-^{14}C and malic acid-4-^{14}C by *L. arabinosus* and NADP-malic enzyme is compared in Table III. The *in vitro* enzymic method showed rapid and specific removal of

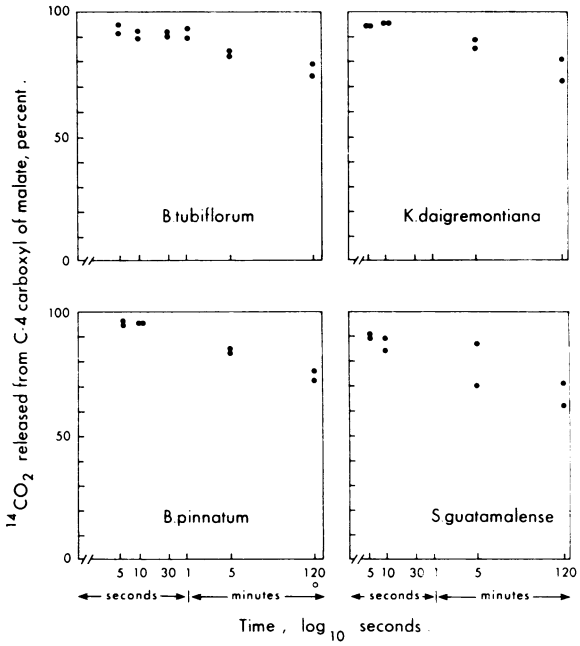

FIG. 3. Degradation of labeled malic acid isolated from four species of Crassulacean plants after dark exposure to $^{14}CO_2$ for various times. Exposure times are plotted on the abscissa on a log seconds scale: actual times are shown. Degradation was by the *in vitro* NADP-malic enzyme method. Values shown are percentage of radioactivity in the C-4 carboxyl at the indicated time. Each point represents the mean of duplicate assays of one experiment.

Table II. *Degradation of Labeled Malic Acid Isolated from B. tubiflorum after Exposure to $^{14}CO_2$ in Dark or Light for 5 Min at Various Stages of the Diurnal Cycle*

Degradation was by the *in vitro* NADP-malic enzyme method.

Stage of Diurnal Cycle Harvested	Malic Acid Content	^{14}C in C-4 of Malic Acid Following 5 Min Exposure	
		Dark	Light
	μeq/g	%	
End of light period	39	84	57
3 hr dark	88	85	69
12 hr dark	174	93	73
End of light period	38	80	62

Table III. *Comparison of Degradation of Malic Acid-^{14}C by Lactobacillus arabinosus and by NADP-Malic Enzyme*

The assays contained 0.2 μmole of malic acid and other components as specified in "Materials and Methods."

Reaction Time	^{14}C Released as $^{14}CO_2$			
	Lactobacillus		NADP-Malic enzyme	
	4-^{14}C	U-^{14}C	4-^{14}C	U-^{14}C
min		%		
10	63	21	96	27
20	67	23	96	27
60	71	24		
120	78	36		
240	80	39		

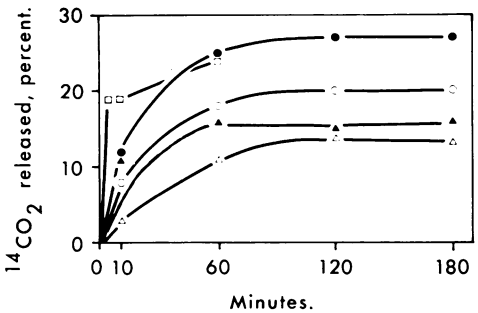

FIG. 4. Degradation of malic acid-U-^{14}C by *L. arabinosus* at different substrate concentrations, otherwise under standard assay conditions. Percentage of radioactivity released as $^{14}CO_2$ is shown against elapsed reaction time. □: 2 μmoles of malate per 10 mg dry wt of bacteria; ●: 20 μmoles; ○: 40 μmoles; ▲: 80 μmoles; △: 100 μmoles.

the C-4 carboxyl from malic acid-^{14}C (*cf.* 7). The bacterial degradation underestimated the label in the C-4 carboxyl in short term experiments and released nearly 40% of the label from malic acid-U-^{14}C in the long term. These shortcomings of the *L. arabinosus* method were investigated further.

The age of culture had some effect on the course of $^{14}CO_2$ release from malic acid-^{14}C (17). Cells of *L. arabinosus* harvested after 12, 24, 36, and 48 hr of culture were used in the standard assay and the time course of $^{14}CO_2$ release is shown in Table IV. The results show first, that 12-hr cultures released more than the expected 25% of radioactivity from malic acid-U-^{14}C, and secondly, no culture completely removed the label from malic acid-4-^{14}C. Anaerobiosis (23) had no significant effect on the rate of reaction or the ability of the bacteria to complete decarboxylation of malic acid-4-^{14}C (Table I).

In previous studies high malic acid concentrations were employed during degradation by *L. arabinosus* (5, 16, 23). The effect of varying substrate concentration on the ability of *L. arabinosus* to degrade labeled malic acid was examined using samples of malic acid-U-^{14}C and malic acid-4-^{14}C containing 0.2 μmole of malic acid-^{14}C together with increasing concentrations of unlabeled malic acid under otherwise standard assay conditions. The time course of $^{14}CO_2$ release is shown in Figures 4 and 5. Figure 4 shows that at substrate concentrations in excess of 20 μmoles malic acid per 10 mg bacteria, the ability of the organism to remove 25% of the label from added malic acid was impaired. Even when the *L. arabinosus* assay was not overloaded with substrate (less than 20 μmoles malic acid in assay), the removal of ^{14}C from malic acid-4-^{14}C was far from complete (Fig. 5 and Table IV).

DISCUSSION

The results reported here, using the *in vitro* NADP-malic enzyme method of malic acid degradation, show first, that the malic acid formed in short term exposures of CAM leaves to $^{14}CO_2$ in the dark is greater than 90% C-4 labeled and second, that the proportion of label in the C-4 of malic acid so formed is not constant with time of exposure. These findings conflict with the data of Bradbeer *et al.* (6), Stiller (23), and Jolchine (9), who reported a *constant* 2:1 distribution of label between the C-4 and C-1 carboxyl carbons of malic acid isolated from

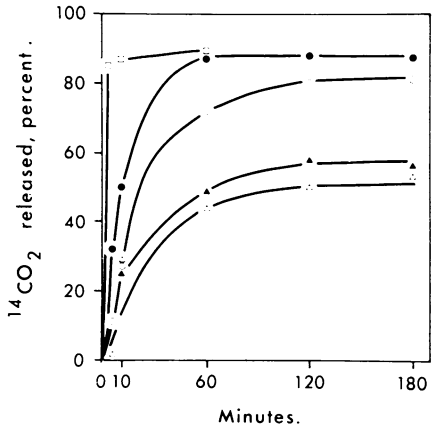

FIG. 5. Degradation of malic acid-4-^{14}C by *L. arabinosus* at different substrate concentrations, otherwise under standard assay conditions; notation as in Figure 4.

Table IV. *Decarboxylation of Malic Acid-4-^{14}C and Malic Acid-U-^{14}C in Standard Assay Conditions by L. arabinosus Cultured for Varying Periods*

Elapsed Reaction Time	Age of Culture, hr							
	12		24		36		48	
	U-^{14}C	4-^{14}C	U-^{14}C	4-^{14}C	U-^{14}C	4-^{14}C	U-^{14}C	4-^{14}C
min	% ^{14}C *released as* $^{14}CO_2$							
0	0	0	0	0	0	0	0	0
3	18	85	19	85	16	76	7	43
6	17	85	19	88	16	86	13	75
9	20	86	19	87	16	86	19	82
60	37	87	24	89	19	89	27	87

CAM tissues after dark $^{14}CO_2$ fixation. These authors used the *L. arabinosus* technique of degrading labeled malic acid.

The degradative application of *L. arabinosus* was developed from the analytical applications described by Blanchard *et al.* (4) and Nossal (16, 17). Thus Bradbeer (5), Aronoff (1), and Avadhani (2) applied the organism for the degradation of malic acid-^{14}C. The experiments reported here show two ways in which the application of malic acid-adapted *L. arabinosus* to the degradation of labeled malic acid can lead to an underestimation of label in the C-4 carboxyl. First, it was shown that when the substrate concentration exceeded 20 μmoles per 10 mg dry weight of bacteria, the β-decarboxylation reaction ceased short of completion. Widely varied ratios of bacteria to substrate have been employed. For example, Blanchard *et al.* (4) used 5 μmoles malic acid per 10 mg bacteria; Nossal (16, 17) used 50, but examined rates of decarboxylation; Bradbeer (5) used 80 to 90, and Stiller (23) used 37 to 50 μmoles per 10 mg bacteria. These latter concentrations could lead to an underestimation of the label in the β-carboxyl carbon. The release of >25% label from malic acid-U-^{14}C in some of our experiments suggests that the bacteria may also degrade the lactate-^{14}C arising from malic acid, leading to an overestimation of the C-4 component.

Second, in our experiments, when the substrate supply was such that β-decarboxylation proceeded to completion (as shown by 25% decarboxylation of malic acid-U-^{14}C), not all the radioactivity was removed from malic acid-4-^{14}C. Blanchard *et al.* (4) demonstrated fumarase activity in *L. arabinosus* equivalent to 5% of the malic enzyme activity. The results presented here suggest that fumarase randomization of label in malic acid-4-^{14}C proceeded to a significant extent. Aging of the culture, suggested by Nossal (17) as a means to overcome this difficulty, was not effective in our experiments.

In several studies, the degradation of malic acid-^{14}C by *L. arabinosus* gave estimates of greater than 90% label in the C-4 carboxyl carbon (18, 19, 21, 27). The malic acid concentration used in most of these assays was low (less than 5 μmoles per 10 mg bacteria). It is also probable that the fumarase activity of different strains or different preparations of *L. arabinosus* may vary, relative to the NAD malic enzyme activity. If the bacteria are to be used for degradative purposes, they must be checked against standards of malic acid-U-^{14}C and -4-^{14}C at appropriate substrate concentrations.

In the absence of these checks and in view of the data obtained in the present experiments, we believe that the constant 2:1 labeling pattern in carboxyl carbons of malic acid-^{14}C isolated during CAM (6, 9, 23) is not a true feature of CAM and is most likely an artifact of the degradation technique employed. We have not degraded the malic acid beyond determining the label in the C-4 carboxyl; the distribution of label in the remaining C_3 moiety is not relevant to the discussion developed here. It should be noted that the combined use of chemical and bacterial procedures by Bradbeer *et al.* (6) convincingly demonstrates that malic acid from CAM remains labeled in the C-1+C-4 carboxyl carbons only.

The demonstration of the 2:1 ratio by Bradbeer *et al.* (6) provided the basis of the two carboxylation-step scheme shown in Figure 1. The present demonstration that the initial fixation product is almost exclusively C-4 carboxyl labeled removes the basis for the scheme and suggests that only one carboxylation step, the β-carboxylation of P-enolpyruvate, is involved in the principal dark fixation of CO_2 in CAM. This view is further supported in recent experiments by M. Kluge (private communication), in which exogenously supplied P-enolpyruvate markedly stimulated dark $^{14}CO_2$ fixation in *K. daigremontiana*, but did not alter the distribution of label in malic acid. This observation is difficult to reconcile with the double carboxylation sequence (Fig. 1).

As a consequence, the involved regulation of the pentose phosphate pathway and glycolysis required to accommodate the two carboxylation steps may be unnecessary. Rather, P-enolpyruvate from glycolysis would seem the likely substrate for malic acid synthesis during starch breakdown in CAM. The very slow movement of label from the C-4 carboxyl to other carbons of malic acid suggests that only a small proportion of recently synthesized malic acid is in equilibrium with tricarboxylic acid cycle malic acid. These data imply effective compartmentation and transport of dark fixation malic acid (25).

The demonstration of a primary β-carboxylation of P-enolpyruvate as the route to malic acid in CAM allows closer analogy to be drawn between CAM and the C-4 dicarboxylic acid pathway of photosynthesis. However, two principal differences should be highlighted. The spatial separation of carboxylation and decarboxylation processes in C_4 photosynthesis contrasts with the temporal separation in CAM (12, 15), and in CAM the carboxylation substrate is formed from stored carbohydrate, whereas in C_4 photosynthesis, P-enolpyruvate is directly regenerated in the photosynthetic sequence (25).

It remains to be shown that the glycolytic process is capable of the sustained diversion of P-enolpyruvate and the maintenance of respiration during starch conversion to malic acid in CAM tissues.

Acknowledgments—We are grateful to Mrs M. G. Anderson and J. M. Rothschild, Research School of Chemistry, for culturing the *Lactobacillus*, to Dr. M. D. Hatch for a sample of malic acid-4-^{14}C and much helpful advice, and to Dr. Irwin P. Ting for his criticisms of the manuscript.

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