Effect of Varying CO₂ Partial Pressure on Photosynthesis and on Carbon Isotope Composition of Carbon-4 of Malate from the Crassulacean Acid Metabolism Plant Kalanchoë daigremontiana
Hamet et Perr.¹

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ABSTRACT

Intact leaves of Kalanchoë daigremontiana were exposed to CO₂ partial pressures of 100, 300, and 1000 microbars. Malic acid was extracted, purified, and degraded in order to obtain isotopic composition of carbon-1 and carbon-4. From these data, it is possible to calculate the carbon isotope composition of newly fixed carbon in malate. In all three treatments, the isotopic composition of newly introduced carbon is the same as that of the CO₂ source and is independent of CO₂ partial pressures over the range tested. Comparison with numerical models described previously (O’Leary 1981 Phytochemistry 20: 553-567) indicates that we would expect carbon 4 of malate to be 4% more negative than source CO₂ if diffusion is totally limiting or 7% more positive than source CO₂ if carboxylation is totally limiting. Our results demonstrate that stomatal aperture adjusts to changing CO₂ partial pressures and maintains the ratio of diffusion resistance to carboxylation resistance approximately constant. In this study, carboxylation and diffusion resistances balance so that essentially no fractionation occurs during malate synthesis. Gas exchange studies of the same leaves from which malate was extracted show that the extent of malate synthesis over the whole night is nearly independent of CO₂ partial pressure, although there are small variations in CO₂ uptake rate. Both the gas exchange and the isotope studies indicate that the ratio of external to internal CO₂ partial pressure is the same in all three treatments. Inasmuch as a constant ratio will result in constant isotope fractionation, this observation may explain why plants in general have fairly invariable ¹³C contents, despite growing under a variety of environmental conditions.

The carbon isotope fractionations which accompany fixation of atmospheric CO₂ during photosynthesis can be used to distinguish between C₃ and C₄ plants (14, 21, 23). C₃ plants discriminate against ¹³C by about 20‰, whereas C₄ plants discriminate by only about 5‰. CAM plants show more complex behavior because of different isotope fractionations associated with daytime and nighttime carbon fixation.

It has often been assumed that carbon isotope fractionations in plants reflect solely the discrimination against ¹³C by ribulose bisphosphate carboxylase in C₃ plants and PEP¹ carboxylase in C₄ plants, but this assumption is not correct. Quantitative modeling suggests that isotopic compositions of plants are affected by the rate of the initial diffusion of CO₂ into the plant and by the small isotope fractionation accompanying this diffusion (7, 14, 22). Plant isotopic compositions can also be affected by isotopically selective losses of ¹³C or ¹²C during respiration, translocation of materials within the plant, and loss of CO₂ back to the atmosphere during refixation in C₄ and CAM photosynthesis.

Whole plant isotopic compositions represent a long-term integration over a complex series of physical and metabolic events. Such compositions reflect carbon fixation pathways (C₃ versus C₄, dark versus light CO₂ fixation), but they are unlikely to provide detailed pictures of either short-term or localized phenomena within plant metabolism. Studies of isotopic compositions of undifferentiated metabolites of a given class (lipids, amino acids, etc.) provide only a modest improvement (4, 5). To circumvent the difficulties associated with whole plant or metabolite pool studies, we have developed an approach to plant isotope fractionations based on measurements of the particular carbon atom within a particular metabolite which was introduced by the carbon fixation process (15). To date, we have studied the isotopic composition of carbon-4 of malate formed during dark CO₂ fixation in CAM plants. This experimental system is useful because malate is accumulated and stored in the vacuole and is not subject to further metabolism until the following light period. After appropriate corrections, this isotopic composition provides the correct basis for evaluating contributions from CO₂ diffusion, dissolution, hydration, and carboxylation.

In the first application of this approach, O’Leary and Osmond (15) measured isotopic compositions of malate in Kalanchoë daigremontiana and Bryophyllum tubiflorum. In both cases, the isotopic composition of carbon-4 of malate (after appropriate corrections) was significantly more positive than whole-plant carbon under conditions of exclusively dark CO₂ fixation and was similar to the isotopic composition of the CO₂ source. Diurnal variations of the isotopic composition of malate were reported in a subsequent publication (16). Comparison of these isotopic composition data with predictions of a model developed by O’Leary (14) revealed that both CO₂ diffusion and carboxylation are important in limiting the rate of dark fixation in these plants.

This new theoretical treatment has permitted an important correlation between the results of isotopic composition measure-

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2 Abbreviations: PEP, phosphoenolpyruvate; VPD, vapor pressure difference.

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ments and the results of gas exchange measurements. The internal vapor phase CO\(_2\) concentration, CO\(_2\)\(_i\), can be calculated from the isotopic measurements and this value can be compared with that obtained from gas exchange. Experiments on \(K.\ daigremontiana\) showed good correlation between the two methods.

If it is true that diffusion and carboxylation jointly limit the rate of dark CO\(_2\) fixation in CAM plants, then it is natural to ask what environmental parameters will change the balance between diffusion and carboxylation. An obvious possibility is external CO\(_2\) concentration. We would predict that as CO\(_2\) concentration increases, diffusion should become less limiting unless stomatal aperture adjusts itself very significantly. Parallel gas exchange studies and isotopic studies at varying CO\(_2\) concentrations provide means for answering this question and for testing the validity of the theoretical treatment. In this paper, we report the effects of a 10-fold variation in CO\(_2\) external partial pressure on photosynthesis, carbon isotope content, and gas exchange in the CAM plant \(K.\ daigremontiana\). The results of this study increase our confidence in the validity of our theoretical treatment and provide important information regarding the role of diffusion in controlling carbon fixation rates.

**MATERIALS AND METHODS**

**Growth of Plants.** Plants were grown in potting soil from a single clone of plants in a nonshaded greenhouse in Canberra, Australia, during the spring and summer of 1979. When about 5 weeks old, the plantlets were transferred to a growth cabinet and were subjected to the following light-dark regime: 10 h, 23°C, 400 \(\mu\)E m\(^{-2}\) s\(^{-1}\), 70% RH light period/14 h, 17°C, 75 to 80% RH dark period. The plants were watered with 5% Hoagland solution on alternate days. The experiments reported here were performed 11 to 14 weeks after the plants were transferred to the growth chamber.

**Gas Exchange Techniques.** The rates of CO\(_2\) and H\(_2\)O vapor exchange were measured for fully expanded, attached leaves enclosed in a 5-L Perspex chamber using equipment described previously (20). CO\(_2\) partial pressure of 100, 330, or 1,000 \(\mu\)bar was achieved and maintained by passing laboratory air through soda lime to remove CO\(_2\); CO\(_2\) from a cylinder was then bled into the air-stream via a needle valve to give the required concentration. Air pressure within the system was monitored manometrically so that an increase or decrease in pressure would deactivate or activate a solenoid which controlled the air flow. The flow rate through the leaf chamber was 1.5 L min\(^{-1}\). The same cylinder of CO\(_2\) was used for all experiments reported here.

Leaves were exposed to 10 h, 600 \(\mu\)E m\(^{-2}\) s\(^{-1}\), 23°C light period and 14 h, 17°C dark period. Under these conditions, leaf temperature was 15 to 16°C in the dark and 23 to 24°C in the light. VPD between the leaf and the air was maintained near 4 mmol m\(^{-2}\) s\(^{-1}\) during the dark for the 100 \(\mu\)bar-treated plants and between 10 and 15 mmol m\(^{-2}\) s\(^{-1}\) during afternoon CO\(_2\) fixation. VPD in the 300 \(\mu\)bar CO\(_2\) treatments was 9 to 10 mmol m\(^{-2}\) s\(^{-1}\) in the dark and 15 to 15 mmol m\(^{-2}\) s\(^{-1}\) during the afternoon; for the 1,000 \(\mu\)bar CO\(_2\) treatment, VPD in the dark and light were about 17 and 27 mmol m\(^{-2}\) s\(^{-1}\), respectively. It was difficult to decrease the VPD in the 1,000 \(\mu\)bar CO\(_2\) treatments because leaf conductance was low and the air was close to saturation.

Leaf-air VPD and CO\(_2\) partial pressure of air entering the chamber were kept constant by manually adjusting the dew point and CO\(_2\) bleed rate every 15 to 30 min. Rates of CO\(_2\)- and water vapor exchange, leaf conductance, air-leaf VPD, and intercellular CO\(_2\) partial pressure were calculated according to Cowan (2).

Leaves harvested at the end of the light period had been in the chamber for two light periods and the intervening dark period, whereas leaves harvested at the end of the dark period had been in the chamber for two consecutive light-dark cycles.

**Leaf Area Determination.** Upon removal from the gas exchange system, the outline of each leaf was traced on paper. The tracing was photocopied and the copy was excised, weighed, and the leaf area calculated from a standard curve.

**Malate Extraction.** After removal of the lower epidermis and midrib, leaves were homogenized in 80% (v/v) methanol for 60 s at full speed in a Sorvall Omnimixer. The extract was reduced to about 50% of the initial volume by boiling. The resulting slurry was centrifuged for 20 min at 0°C at 10,000g, the supernatant was decanted, and the precipitate was resuspended in H\(_2\)O and respun. The combined supernatant was dried by rotary evaporation under reduced pressure at 50°C. The dry, crimson extract was respun in 1.5 ml H\(_2\)O, transferred to a centrifuge tube, and then brought to 10 ml with ethanol. The protein precipitate was removed by centrifugation and the supernatant was dried as before. The residue was resuspended in 10 ml H\(_2\)O and was stored at -20°C after the malate content was determined.

**Malate Purification.** Malate was purified by ion exchange chromatography. Basic compounds were removed by a cation exchange column (1 x 12 cm Dowex 50W X-8, H\(^+\) form). The eluant was loaded onto an anion exchange column (1 x 30 cm Dowex AG-1, formate form). Malate was separated from other organic acids and from neutral compounds on a 300 ml 0 to 6 N formic acid gradient at a flow rate of 80 ml h\(^{-1}\). The eluant was collected in 3.5-ml fractions. Malate eluted in the vicinity of 3 N HCOOH. The malate-containing fractions were pooled, reduced to about 20% of their initial volume by rotary evaporation, and lyophilized to dryness. The dry extracts were stored at -20°C in the dark until needed.

**Malate Determination.** Malate was identified by HPLC. Reverse-phase HPLC identification was performed using a Waters C\(_{18}\) \(\mu\)Bondapack column (30 cm long, 7.8 mm i.d.) equilibrated with 10 mm KH\(_2\)PO\(_4\), pH 2.5. Flow rate was 1.5 to 2.0 ml min\(^{-1}\) and malate was detected using a refractive index detector. For ion exchange HPLC, malate was identified using a Bio-Rad anion exchange column fitted with a Bio-Rad micro-guard pre-column. The column was equilibrated with 0.013 N H\(_2\)SO\(_4\) at 950 p.s.i. Organic acids were detected by their UV A at 210 nm. Accurate malate quantitation was performed enzymically. Less than 100 nmol malate was added to a 3-ml cuvette containing 0.2 M N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid—NaOH, pH 7.5, 4 mM MnCl\(_2\), 0.5 IU ml\(^{-1}\) malic enzyme, and 1 mM NADP. The change in \(A_{540}\) at endpoint was used to calculate malate content.

**Analysis of Malate.** Carbon-4 of malate was converted to CO\(_2\) using malic enzyme and was recovered on a vacuum line as described elsewhere (15).

**Isolation of Carbons 1 plus 4 of Malate.** Carbons 1 plus 4 of malate were converted to CO\(_2\) enzymically in the following manner.

Fifty amol malate was placed in a flask with 10 ml of CO\(_2\)-free solution containing 0.2 M N-morpholinopropanesulfonic acid (pH 7.1), 4 mM MnCl\(_2\), 100 mM (NH\(_4\))\(_2\)SO\(_4\), 20 mM oxidized GSH, 4 \(\mu\)M thiamine pyrophosphate, 0.5 units malic enzyme, 20 units GSH reductase, and 10 units pyruvate decarboxylase. The reaction was initiated by addition of 1 mM NADP and allowed to proceed for at least 2 d at 25°C. (It is important that the pH of this reaction remain in the region 7.0 to 7.2 because below pH 7.0 the malic enzyme reaction becomes slow, whereas above pH 7.2 the pyruvate decarboxylase reaction becomes slow.) The solution was then acidified with 1 ml concentrated H\(_2\)SO\(_4\), and CO\(_2\) was recovered.

At the termination of each reaction, an aliquot of the acidified solution was neutralized with KOH and assayed for pyruvic and pyruvic acids. It is crucial that the reactions proceed to greater than 99% completion; otherwise, the malic enzyme in the reaction will discriminate against \(^{12}\)C (8) and the CO\(_2\) released during the decarboxylation will be depleted in \(^{13}\)C.

**Carbon Isotope Measurements.** Carbon isotope composition of
CO₂ was measured using a Nuclide RMS6-60 isotope ratio mass spectrometer. The ratios were corrected for ¹⁸O content and machine and leak fractionations. The ratios were converted to δ¹³C values by comparison with standard CO₂ which had been calibrated against a Pee Dee belemnite standard (3).

Calculations. The isotopic composition of carbon-4 of malic acid isolated from _K. daigremontiana_ in the morning reflects not only the isotopic composition of carbon-4 of newly synthesized malic acid but also reflects the isotopic composition of the small pool of malic acid which remained at the end of the previous day. In addition, carbons 1 and 4 of malic acid are to some extent randomized by the action of fumarase following carboxylation (1, 6, 10). Provided that fumarase randomization is incomplete, it is possible to use the isotopic compositions of carbons 1 and 4 to calculate the isotopic composition prior to randomization.

Correction for residual malate is made first. The experimentally determined isotopic compositions of carbon-4 in the morning and evening malate samples will be called δ(4)_{morn} and δ(4)_{even}, respectively, and the corresponding combined isotopic compositions for carbons 1 and 4 will be called δ(1+4)_{morn} and δ(1+4)_{even}, respectively. Using a prime to indicate material newly synthesized during the night,

\[
\delta'(4) = \delta(4)_{morn} - F \delta(4)_{even} / (1 - F)
\]

where F is the proportion of total malate extracted which is present at the beginning of the night and 1 - F is the proportion which is formed during the night. An equation analogous to equation 1 can be applied to the combined isotopic composition of carbons 1 and 4.

Isotopic compositions which have been corrected for randomization by fumarase will be denoted by double primes. Let R be the fraction of malate which has been randomized by fumarase. Of the newly formed malate pool, a fraction R of the molecules will have the same isotopic composition at carbon-1 and carbon-4; the remaining molecules (fraction 1 - R) will have the same isotopic composition as before randomization. The isotopic composition of carbon-4 after correction for fumarase randomization is given by:

\[
\delta''(4) = \delta'(4) / (1 - R) - R \delta'(1+4) / (1 - R)
\]

and the isotopic composition of carbon-1 is given by:

\[
\delta''(1) = 2 \delta'(1+4) - \delta''(4)
\]

Thus, the isotopic composition δ''(4) represents the isotopic composition of carbon-4 of newly synthesized malate, corrected for residual malate, and corrected for fumarase randomization. In cases where it is necessary or desirable to correct for incorporation of respired carbon into newly synthesized malate, a further correction of this isotopic composition will be necessary.

**Calculation of Isotope Effect k¹²/k¹³ and Partitioning Factor k₄/k₅.** The overall isotope effect for incorporation of atmospheric CO₂ into carbon-4 of malate can be calculated from the isotopic composition of the source CO₂, δ(source), and δ''(4) by use of the equation:

\[
k¹²/k¹³ = 1 / [1 - (\delta(source) - \delta''(4)) / (1000 + \delta(source))] (4)
\]

and the partitioning factor, k₄/k₅, can be derived from:

\[
k¹²/k¹³ = E_1(E_2/k_3/k_5)/(1 + k_2/k_5) (5)
\]

Equation 6 was derived by O'Leary (14) for a pathway proposed for atmospheric CO₂ entering the leaf and its incorporation into malate (Fig. 1). E₁ = k₁/k₁', E₂ = k₂/k₂', and E₃ = k₃/k₅. The kinetic fractionations of these steps at 17°C are 1.0044, 1.0044, and 0.9933. Note that E₃ contains the fractionations due to CO₂/HCO₃⁻ interchange and PEP carboxylase. The equation can thus be restated as:

\[
k₃/k₂ = (0.9933 - k¹²/k¹³) / (k¹²/k¹³ - 1.0044) (6)
\]

**Calculation of Internal CO₂ Concentration, CO₂(i).** CO₂(i) was derived from gas exchange measurements by using the relationship:

\[
CO₂(i) = CO₂(external) - 1.6 PA/g (7)
\]

Where CO₂ partial pressures are expressed in μbar, P is the atmospheric pressure [μbar], A is the CO₂ assimilation rate (μmol m⁻² s⁻¹), g is leaf conductance to vapor transfer (μmol m⁻² s⁻¹), and the factor 1.6 is the ratio of the diffusivities of water vapor and CO₂ in air (2).

The average CO₂(i) was calculated from isotopic measurements using the equation (14):

\[
CO₂(i) = CO₂(external) / (1 + k₃/k₂) (8)
\]

Because the value of CO₂(i) thus derived is an average of CO₂(i) over the entire dark period, and gas exchange values are for single time-point measurements, to compare the two it is necessary to calculate the average CO₂(i) over the entire dark period for the gas exchange measurements, weighted according to the carbon assimilation rate.

**RESULTS**

Intact leaves of _K. daigremontiana_ were exposed to 100, 330, or 1,000 μbar CO₂ in an open gas exchange system. After a 36 to 48 h equilibration, CO₂ and H₂O exchange, leaf temperature, leaf conductance, leaf-air vapor pressure difference, CO₂ (external) partial pressure, and CO₂ (internal) partial pressure were measured or calculated. Two complete series of measurements on separate leaves were made at each CO₂ concentration. Duplicate measurements at a particular concentration produced very similar results.

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**Fig. 1.** Pathway for incorporation of external CO₂ into malate during dark CO₂ fixation in _K. daigremontiana_. All steps except carboxylation are potentially reversible. k₁, k₂, and k₃ are the rate constants for diffusion in, diffusion out, and carboxylation, respectively (Eq. 5).
were harvested internal calculated set the combined a each leaves were to partial randomization leaves. That resist for residual malate. Yields of malic and total amounts of CO₂ absorbed during each treatment are summarized in Table I.

Decarboxylation of malic acid using malic enzyme provided the isotopic composition of carbon-4 of this material. Decarboxylation using a mixture of malic enzyme and pyruvate decarboxylase provided the combined isotopic composition of carbons 1 and 4. Isotopic compositions obtained from duplicate measurements at each CO₂ concentration are summarized in Table II. Also included in Table II are isotopic compositions for whole-leaf carbon obtained by combustion analysis. The δ^{13}C value for the CO₂ used in these experiments is −15.9‰.

The isotopic composition which is needed to calculate the isotope fractionation associated with carbon fixation is the isotopic composition of carbon-4 of newly formed malate. To obtain this value, the measured isotopic composition of carbon-4 of malic acid from the end of the dark period must be corrected for residual malic acid present at the beginning of the dark period and for partial randomization of carbons 1 and 4 by fumarase (cf. "Materials and Methods"). Both the raw data and the isotopic compositions obtained after each correction are shown in Table II, along with the final corrected isotopic compositions.

**DISCUSSION**

**Effect of CO₂ Partial Pressure on CO₂ and Water Exchange.**

Variation of CO₂ partial pressure between 100 and 1,000 μbar produced few changes in the extent and pattern of CO₂ exchange in the dark (Figs. 2–4). The rate of dark CO₂ uptake was lower in the 100 μbar than in the 300 or 1,000 μbar treated leaves, but the leaves in the 100 μbar treatment continued to assimilate carbon at the maximum rate for a longer period. Consequently, over the entire dark period, net CO₂ uptake was similar for all treatments (Table I).

Despite the similarity of the patterns and rates of CO₂ assimilation during the dark, leaf conductance showed a 10-fold variation. Conductance was only about 15 mmol m⁻² s⁻¹ in the 1,000 μbar treated leaves compared to 100 mmol m⁻² s⁻¹ in the 330 μbar treated leaves and 150 mmol m⁻² s⁻¹ in the 100 μbar treated leaves. At higher CO₂ levels, the CO₂ assimilation rate can clearly be maintained at a lower conductance. This decrease in conductance with increasing CO₂ partial pressure results in an increase in water use efficiency from 60 g H₂O g⁻¹ CO₂ in the 100 μbar treated leaves to 41 g H₂O g⁻¹ CO₂ in the 330 μbar treated leaves and 19 g H₂O g⁻¹ CO₂ in the 1,000 μbar treated leaves. Similar increases in water use efficiency in C₃ and C₄ plants with increasing CO₂ levels have been observed by Wong et al. (24, 25).

In all treatments, the increase of assimilation rate at the beginning of the dark period is accompanied by an increase in conductance. However, during this period there is a rapid drop in CO₂, which indicates that the capacity of the leaves to assimilate CO₂ increases more rapidly than the capacity for CO₂ diffusion into the leaves. That is, stomatal resistance appears to change in response to increasing assimilation rate. After about 2 h, assimilation rate and stomatal resistance reach a steady state and there
is little further change for several h. At the end of the night, the rate of CO₂ uptake decreases. This appears to happen at different times in the different treatments in response to accumulation of more or less the same amount of malate in every treatment. Thus, it appears that the extent of malate accumulation is controlled by innate factors in the plant, rather than by CO₂ levels. During the cessation of CO₂ fixation, there is an increase in CO₂(i), which indicates that the capacity of the leaves to assimilate CO₂ is decreasing more rapidly than stomatal conductance is changing. This sequence of events is parallel to that observed at the beginning of the night and again indicates that stomatal conductance follows photosynthetic rate. Thus, it appears to be carboxylation capacity (i.e. activity of PEP carboxylase or synthesis of PEP) rather than stomatal aperture that leads to inhibition of carbon fixation at the end of the night.

External CO₂ concentration has a greater effect on daytime CO₂ fixation than on nighttime CO₂ fixation. Leaves exposed to 330 or 1,000 μbar CO₂ exhibited considerable afternoon uptake at maximum rates approximately 0.7 times the maximum rate of dark uptake (Figs. 3 and 4). At 100 μbar CO₂, on the other hand, there is little afternoon CO₂ assimilation (Fig. 2). The maximum rate was less than one-eighth that observed during the dark. In spite of the difference in afternoon CO₂ uptake between the 100 and 330 μbar treated leaves, leaf conductances were similar (50–85 mmol m⁻² s⁻¹). The lack of significant CO₂ fixation at 100 μbar supports the proposition (17, 18) that afternoon fixation occurs solely by ribulose bisphosphate carboxylase, rather than by PEP carboxylase.

**Isotopic Composition of Malate.** Replicate determination of the isotopic composition of malic acid for different leaves of a given treatment shows the same high reproducibility seen in our previous work (15). Although the sample size is too small to permit adequate statistical analysis, previous experience indicates that the uncertainty in our isotopic measurements is less than 1%.

The isotopic analysis in the present experiments is complicated by the fact that the plants used had been grown to maturity in a normal atmosphere (330 μbar CO₂, δ¹³C = −7‰) and thus had acquired a normal whole leaf δ¹³C value (−15‰). Two d prior to the beginning of the gas exchange studies, the plants were transferred to the desired atmosphere (various CO₂ partial pressures, δ¹³C = −15.9‰). Two principal lines of evidence indicate that the isotopic analyses are still correct in spite of these complications. In the first place, the calculated δ¹³C value for carbon-4 of malate after correction in the 330 μbar treatment corresponds to that seen in our previous work; that is, it is the same as that of the CO₂ source. Second, the calculated isotopic compositions for carbon-1 of malate are as expected. This carbon arises from starch and thus reflects the long-term isotopic history of the plant as well as the recent isotopic history. A δ¹³C value near −7‰ is consistent with that seen in other studies not complicated by a change in the δ¹³C value of the source CO₂.

In our earlier studies of *K. daigremontiana* (15, 16), the isotopic composition of carbon-1 of malate was obtained by an indirect procedure involving combustion of whole malate, rather than by the double decarboxylation procedure used here. It is encouraging that the same isotopic composition for carbon-1 is obtained by
either procedure.

The most significant consequence of the change in $\delta^{13}C$ value of the source CO$_2$ in these studies is the resulting difference in isotopic compositions between carbon-1 and carbon-4 of malic acid. Inasmuch as the isotopic information contained in these two sites becomes somewhat scrambled because of the action of fumarase, the adequacy of the correction for fumarase randomization should be carefully examined. A number of studies in other laboratories (1, 10) and in our own (unpublished) have consistently
given randomization factors near two-thirds for malate in K. daigremontiana, and this is the figure we have used. If this figure were not exactly correct, then the quantitative conclusions regarding the relative rates of the diffusion and carboxylation processes would be slightly changed, but the qualitative picture would not be changed. The major conclusion of this study, that the partitioning of the internal CO$_2$ pool is independent of external CO$_2$ partial pressure, would only be altered if the extent of fumarase randomization varied with external CO$_2$ partial pressure.

In our previous studies of K. daigremontiana (15), we corrected the isotopic composition of carbon-4 of malate for the fact that some of the fixed carbon arises from respiration, rather than from the atmosphere. We have not made this correction in the present studies for the following reason. The isotopic composition of dark-respired CO$_2$ in CAM plants is not well known, but it is believed to be similar to the isotopic composition of the whole leaf from which it comes (14, 19). Thus, in the present studies, we would expect that respired carbon has a $\delta^{13}C$ near $-15\%$o, which is similar to the $\delta^{13}C$ value for external CO$_2$ $-15.9\%$o. The amount of respired carbon is small (11) and only a fraction of this carbon is refixed (15). Thus, we expect that less than 10% of the malate synthesized during the dark period arises from respired carbon, and the change in the isotopic composition of carbon-4 of malate from this source should be no more than 0.2%.

Interpretation of Isotope Fractionation Data. The most striking feature of the isotopic composition data obtained in this study is that after correction, the isotopic composition of carbon-4 of malic acid synthesized during dark CO$_2$ fixation in K. daigremontiana is

Table 1. Nocturnal Change in Malate Content of Leaves of K. daigremontiana Exposed to CO$_2$

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<tr>
<th>External CO$_2$ Partial Pressure</th>
<th>Malate</th>
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<tr>
<td>µbar</td>
<td>µmol g$^{-1}$ fresh wt</td>
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<tr>
<td>100 (see Fig. 2)</td>
<td>80.7</td>
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<tr>
<td>100</td>
<td>No sample</td>
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<tr>
<td>330 (see Fig. 3)</td>
<td>80.3</td>
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<tr>
<td>330</td>
<td>71.5</td>
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<tr>
<td>1,000 (see Fig. 4)</td>
<td>61.5</td>
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<tr>
<td>1,000</td>
<td>70.5</td>
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the same, within experimental error, as that of the atmospheric CO₂ source. Further, this correspondence holds independent of the atmospheric CO₂ partial pressure at least over the range from 100 μbar to 1,000 μbar. Results at 330 μbar are similar to those reported previously (15).

When *K. daigremontiana* is exposed to CO₂ of δ¹³C value −7‰ in the dark and is maintained in a CO₂-free atmosphere during the light period, the plant has a δ¹³C value of −11‰ (12). Our results show that initially fixed carbon under these circumstances has a δ¹³C value of −7‰, thus, some process subsequent to the initial carbon assimilation must contribute to the isotopic composition of the plant. One likely source of this isotopic shift is fractionation accompanying CO₂ loss during deacidification. Some CO₂ is lost back to the atmosphere during deacidification. (Figs. 2–4), but its isotopic composition has not been measured to date.

The pathway for nighttime carbon fixation in *K. daigremontiana* is shown schematically in Figure 1. External CO₂ first diffuses through the stomata into the intercellular air spaces. This internal CO₂ (which corresponds to CO₂(i) of our earlier discussion) may either diffuse back to the atmosphere or else may be dissolved in the cell sap, converted via carbonic anhydrase into HCO₃⁻, and then converted into oxaloacetate by means of PEP carboxylase. Reduction of oxaloacetate produces malate, which is the product under study. If we assume, as we have done previously (14), that the principal factors which control the rate of CO₂ incorporation during the dark are diffusion of CO₂ into the leaves and absorption of CO₂ by PEP carboxylase, then we can calculate the partitioning ratio k₂/k₃, which is the quotient of the flux of CO₂(i) through PEP carboxylase divided by the flux of CO₂(i) back to the atmosphere. This ratio, together with the calculated isotope fractions, is given in Table III. An immediate consequence of the constancy of the isotopic compositions is that the ratio k₂/k₃ is independent of CO₂ concentration. In all cases, CO₂ in the internal pool is carboxylated 1.5 to 3.3 times more rapidly than it returns to the atmosphere. Thus, there is a substantial diffusional limitation to dark fixation in *K. daigremontiana*, even at 1,000 μbar CO₂. This constancy of k₂/k₃ is achieved by changes in leaf conductance. As described previously, leaf conductance is 10-fold lower in the 1,000 μbar treated leaves than in the 100 μbar treated leaves (Figs. 2 and 4). These changes are apparent both from the gas exchange studies and from the isotope studies.

Our numerical modeling of the isotope results also permits calculation of CO₂(i), the internal gas phase CO₂ concentration (Table IV). The isotope results indicate that the ratio between the internal and the external CO₂ concentrations is constant, independent of the absolute CO₂ level. The same result is obtained from the gas exchange measurements (Table IV), but there is a consistent difference between the gas exchange data and the isotope data: the isotope data give internal CO₂ concentrations that are lower than those calculated from gas exchange. A likely

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<th>CO₂ Treatment (μbar) at Different Harvest Times</th>
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<tr>
<td>even</td>
</tr>
<tr>
<td>Carbon-4</td>
</tr>
<tr>
<td>Carbon-1+4</td>
</tr>
<tr>
<td>δ¹³C (%) observed</td>
</tr>
<tr>
<td>Carbon-4</td>
</tr>
<tr>
<td>Carbon-1+4</td>
</tr>
<tr>
<td>δ¹³C (%) after correction for residual malate</td>
</tr>
<tr>
<td>Carbon-4</td>
</tr>
<tr>
<td>Carbon-1</td>
</tr>
<tr>
<td>δ¹³C (%) after correction for fumarase randomization</td>
</tr>
<tr>
<td>Source CO₂</td>
</tr>
<tr>
<td>Whole leaf</td>
</tr>
</tbody>
</table>

Table IV. Ratio of External to Internal CO₂, Calculated from Gas Exchange Measurements and from Isotopic Measurements, in Leaves of *K. daigremontiana* Exposed to CO₂

<table>
<thead>
<tr>
<th>CO₂ (ext)</th>
<th>Gas Exchange Measurements</th>
<th>Isotope Measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>μbar</td>
<td>ratio</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.8</td>
<td>2.6</td>
</tr>
<tr>
<td>330</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
<td>330</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>1,000</td>
<td>1.5</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table III. Overall Isotope Effect, k⁺¹³/k⁻¹³, and Partitioning Factor, k₂/k₃, for CO₂ (ext) Incorporated into Malate during the Dark by Leaves of *K. daigremontiana* Exposed to CO₂

<table>
<thead>
<tr>
<th>CO₂ (ext)</th>
<th>δ¹³C/k⁻¹³</th>
<th>k₂/k₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>μbar</td>
<td>ratio</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.0002</td>
<td>1.6</td>
</tr>
<tr>
<td>330</td>
<td>1.0002</td>
<td>1.6</td>
</tr>
<tr>
<td>330</td>
<td>1.0000</td>
<td>1.5</td>
</tr>
<tr>
<td>1,000</td>
<td>1.0007</td>
<td>2.0</td>
</tr>
<tr>
<td>1,000</td>
<td>1.0019</td>
<td>3.3</td>
</tr>
</tbody>
</table>
The spectrometer.

Fractionation.

Not to the present data the kinetic distance are very diffusion must occur, but the absolute rates of diffusion (per unit distance) are very different in the two phases. Carboxic anhydrase is present in CAM plants, so CO₂ hydration should be at or near equilibrium (22).

The mathematical treatment needed to include additional steps in the model is available (14), but unfortunately the magnitudes of the carbon isotope fractionations associated with these steps are not known. Liquid phase diffusion has generally been assumed not to fractionate isotopes, but this might not be so. Expansion of the kinetic treatment to include liquid phase diffusion increases the number of parameters sufficiently that a unique fit to the present data is not possible. Nevertheless, several trends are apparent. First, the role of stomatal diffusion in limiting carbon fixation rates will be only slightly altered if liquid diffusion is important. Second, the constancy of the ratio CO₂(l)/CO₂(ext) is likely to be preserved. Inclusion of a modest contribution from liquid phase diffusion has the effect of increasing the CO₂(l) values calculated from the isotopic data and bringing them into line with the values obtained from gas exchange. Whether this does in fact indicate that liquid phase diffusion is important is not clear.

The effect of CO₂ partial pressure on isotopic compositions of plants has not yet been examined in detail except for our studies of K. daigremontiana. Gas exchange studies of C₃ and C₄ plants have revealed the same general phenomenon seen here, namely, the tendency for leaves to keep the ratio CO₂(l)/CO₂(ext) constant. Since a constant ratio results in constant isotope fractionation, this observation may explain why plants have fairly invariable ^1³C contents, despite growing under a large variety of environmental conditions.

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