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Temperature Dependence of Carbon Isotope Fractionation in CAM Plants[1]

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ELIANE DELEENS, ISABEL TREICHEL, AND MARION H. O'LEARY*
Departments of Chemistry and Biochemistry, University of Wisconsin, Madison, Wisconsin 53706 (I.T., M.H.O'L.); *and Institut de Physiologie Vegetale, Gif sur Yvette, France* (E.D.)

ABSTRACT

The carbon isotope fractionation associated with nocturnal malic acid synthesis in *Kalanchoë daigremontiana* and *Bryophyllum tubiflorum* was calculated from the isotopic composition of carbon-4 of malic acid, after appropriate corrections. In the lowest temperature treatment (17°C nights, 23°C days), the isotope fractionation for both plants is −4‰ (that is, malate is enriched in ^{13}C relative to the atmosphere). For *K. daigremontiana*, the isotope fractionation decreases with increasing temperature, becoming approximately 0‰ at 27°C/33°C. Detailed analysis of temperature effects on the isotope fractionation indicates that stomatal aperture decreases with increasing temperature and carboxylation capacity increases. For *B. tubiflorum*, the temperature dependence of the isotope fractionation is smaller and is principally attributed to the normal temperature dependences of the rates of diffusion and carboxylation steps. The small change in the isotopic composition of remaining malic acid in both species which is observed during deacidification indicates that malate release, rather than decarboxylation, is rate limiting in the deacidification process.

The fractionation of carbon isotopes which occurs during photosynthesis reflects photosynthetic pathways (20). When carbon fixation occurs via PEP[2] carboxylase, the fractionation is small (typically leaf $\delta^{13}C = -11$ to -14‰). When carbon fixation occurs via RuBP carboxylase, the fractionation is large[3] (leaf $\delta^{13}C = -25$ to -30‰). These fractionations have multiple causes. The carboxylation step itself is a principal contributor, but stomatal diffusion and a number of other processes also contribute.

Carbon isotopic compositions of whole plants provide only an overall, long-term view of carbon metabolism. We have developed a method that enables us to look at the initial steps in carbon fixation in CAM plants during the course of a single night (9, 22). At night, CAM plants use PEP carboxylase and malate dehydrogenase to synthesize malic acid, which is then stored in large quantities in the vacuole. We purify this malate and measure the isotopic composition of carbon-4 (the carbon which arises from atmospheric CO_2). We make corrections for fumarase randomization, for residual malate from the end of the previous day, and for carbon derived from respiration. The isotope fractionation thus calculated is independent of steps subsequent to malate synthesis and independent of any contributions from direct C_3 carbon fixation.

In parallel with these studies, we have developed a mathematical model of the isotope fractionation process (9, 20, 22). Comparison of theory with experiment reveals that the isotope fractionation associated with malate synthesis in CAM plants reflects a balance between stomatal conductance and carboxylation capacity. In cases examined to date, neither carboxylation nor diffusion is entirely limiting. ^{18}O Tracer studies have been used to demonstrate that the activity of carbonic anhydrase is several-fold higher than that of PEP carboxylase.

In the case of *K. daigremontiana*, the isotope fractionation associated with malate synthesis is independent of CO_2 concentration. It appears that stomatal aperture decreases as CO_2 concentration increases, and the ratio of external CO_2 concentration to internal CO_2 concentration stays constant, as does the partitioning of the internal CO_2 pool (9). Results obtained from isotopic studies have been correlated with results of gas-exchange studies (7, 20).

It has been known for some time that nocturnal CO_2 fixation contributes decreasingly to net CO_2 fixation in CAM plants at higher temperatures (14, 15, 25). The purpose of this work is to learn about the temperature dependences of individual steps in the CAM pathway. Because our method focuses specifically on malate synthesis, we can learn about how rates of diffusion and carboxylation change as the temperature increases. We also present data concerning changes in the isotopic composition of malate during deacidification which tell about the mechanics of the deacidification process.

MATERIALS AND METHODS

Plants of *Kalanchoë daigremontiana* and *Bryophyllum tubiflorum* were grown from leaflets in a well-ventilated chamber in the University of Wisconsin Biotron with a 10-h light period and a 14-h dark period. The air in the Biotron circulates freely, with a complete turnover in 4 min; Its $\delta^{13}C$ value is −8‰. The plants were watered on alternate days with water and with half-strength Hoagland solution. Three temperature regimes were investigated: the first set of experiments was conducted with 5-month-old plants with a 17°C night temperature and a 23°C day temperature. After sample collection was complete, the temperature was changed to 22°C/28°C and the plants were allowed to adapt for 10 d before samples were taken. After samples were taken at the second temperature, the temperature was changed to 27°C/33°C and the plants were allowed to adapt for 10 d before samples were taken.

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[2] Abbreviations: PEP, phosphoenolpyruvate; RuBP, ribulose bisphosphate.

[3] $\delta^{13}C$ is defined by the equation

$$\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000$$

where R is the ratio $^{13}CO_2/^{12}CO_2$ derived from the mass spectrometer measurements. A more positive value of $\delta^{13}C$ means that the sample contains more ^{13}C. Isotope fractionation = $\delta_{source} - \delta_{product}$.

Leaf samples were collected at the time the lights were turned on (8:30 AM), 2 h after the lights were turned on (10:30 AM), at which time about one-third of the original malate had been metabolized, 4.5 h after the lights were turned on (1:00 PM), at which time a little more than half of the original malate had been metabolized, and when the lights were turned off (6:30 PM). For each treatment, four independent samples from different plants were analyzed.

For study of the isotopic composition of respired CO_2, four plants were enclosed in narrow Lucite boxes overnight, and the leaves were allowed to re-absorb the CO_2 released by respiration.

Immediately following sample collection, the leaf midrib was removed and the leaf was weighed and homogenized in 80‰ methanol and malate was purified by ion-exchange chromatography (9). Reported yields of malate (Tables I and II) are based on quantities of purified malate, analyzed with malic enzyme (9). Previous studies have demonstrated that under these conditions the quantity and isotopic integrity of malate are maintained throughout the procedure (22).

Carbon-4 of malate was removed by treatment of the purified material with malic enzyme, and carbons 1 and 4 were removed by simultaneous treatment with malic enzyme and pyruvate decarboxylase (9). In all cases, reaction solutions were checked after the decarboxylation was complete in order to ensure that no malate remained (such would lead to spurious isotope results). CO_2 was purified under high vacuum.

Isotope ratios were measured on a Finnigan Delta-E isotope-ratio mass spectrometer. Values have been corrected for instrumental effects and for the presence of ^{17}O (3) and are reported as $\delta^{13}C$ relative to the usual Pee Dee Belemnite standard. Carbon isotope composition of whole leaves was measured using the combustion method previously described (4). Values of $\delta^{13}C$ are estimated to be accurate to ±0.4‰.

THEORY

It is generally accepted that malic acid is the predominant product of nocturnal CO_2 fixation in CAM plants (14, 24). Malic acid isolated at 8:30 AM in our experiments is principally formed by overnight synthesis occurring by way of PEP carboxylase and malate dehydrogenase. Previous studies of *K. daigremontiana* have established that there is a 1:1 correspondence between CO_2 uptake and malate synthesis (15, 22). Carbon-4 of this material is derived from CO_2, and to a first approximation the isotope fractionation associated with CO_2 fixation is obtained simply from the difference between atmospheric CO_2 and this carbon. However, several small corrections to the isotopic composition of carbon-4 of malate are necessary. The complete rationale of the experiment and the detailed mathematical treatment have been described (9). Briefly, some of the malate (generally 10–20%) is not newly synthesized, but instead remains from the end of the previous light period. The quantity and isotopic composition of this malate are obtained by study of samples collected at 6:30 PM. Second, carbons 1 and 4 of malate in CAM plants are partially randomized by fumarase (24). The isotopic compositions of carbon-4 and carbon-1 of malate prior to the randomization can be calculated from the measured isotopic compositions and the extent of randomization. The extent of randomization of malate has often been observed to be near two-thirds (24). Recent unpublished measurements in our laboratory are consistent with this observation for all cases studied here except *B. tubiflorum* in the low temperature regime, where the extent of randomization is near 50%. The mathematical form of these first two corrections has been described previously (9). Third, a small amount (5–20%) of the CO_2 in the new malate comes not from atmospheric CO_2, but instead from respiration during the night. The correction for respiration is made by use of an equation analogous to equation 1 of Holtum *et al.* (9). The amount of carbon derived from respiration is assumed to be one-third of the amount collected in the CO_2-free respiration experiment.[4]

Table I. *Observed $\delta^{13}C$ Values and Malate Quantities for K. daigremontiana*

Temperature Regime	$\delta^{13}C$			Malate
	C-4	C-1 + C-4	Whole leaf	
time		‰		*μmol/g fresh wt*
17/23				
6:30 PM	−0.63	−1.79	−17.4, −18.4	11.0
8:30 AM	−4.30	−4.52		79.1
10:30 AM	−5.12	−5.38		52.9
1:00 PM	−5.59	−5.23		37.4
8:30 AM[a]	−4.80	−4.33		25.6
22/28				
6:30 PM	−6.04	−6.11	−15.6, −16.3	17.5
8:30 AM	−6.38	−6.55		64.1
10:30 AM	−6.85	−5.88		48.4
1:00 PM	−5.52	−5.57		34.7
8:30 AM[a]	−5.61	−5.71		32.7
27/33				
6:30 PM	−5.68	−5.78	−15.0, −15.8	14.5
8:30 AM	−7.40	−7.30		72.6
10:30 AM	−6.91	−7.00		53.3
1:00 PM	−6.89	−6.77		42.4
8:30 AM[a]	−6.40	−6.60		26.8

[a] CO_2-free atmosphere.

RESULTS

Isotopic data for *K. daigremontiana* and *B. tubiflorum* are given in Tables I and II, respectively. The isotopic compositions reported here are similar to the more limited data obtained previously (9, 22). Carbon-4 of malate is invariably slightly more positive than the CO_2 from which it is derived ($\delta^{13}C = -8‰$).

In this study, freely watered *K. daigremontiana* and *B. tubiflorum* made large amounts of malate during the night in all three temperature regimes. For the former species, these results are similar to our previous results (22) and to results of Kaplan *et al.* (13) and Medina and Osmond (15), both of whom found at most modest reductions in CO_2 fixation (13) or malate synthesis (15) at higher temperatures. Previous gas exchange studies have shown that although the total amount of malate synthesized overnight varies only slightly with temperature, the time course of CO_2 fixation is temperature dependent, with malate accumulation being more rapid at lower temperature (15).

The $\delta^{13}C$ values of the whole leaves, especially in *B. tubiflorum*, show a trend from more negative at low temperature to more positive at higher temperatures. It is known that high CAM level cannot be achieved on short days when low temperatures are applied to the plants during the first stages of their development (1, 6). The CAM level increased with age, but the growth of the plants remained low; the $\delta^{13}C$ value of the total plant, which integrates C_3 and C_4 metabolism over the entire growth period, cannot vary to a large extent due to the low turnover of the constitutive matter (6). The two higher temperature regimes afforded more rapid growth (as seen by dry weight variations,

[4] We assume that respiratory CO_2 partitions between carboxylation and release to the atmosphere in the same way that the internal CO_2 pool partitions.

Table II. *Observed $\delta^{13}C$ Values and Malate Quantities for B. tubiflorum*

Temperature Regime	$\delta^{13}C$			Malate
	C-4	C-1 + C-4	Whole leaf	
time		‰		*μmol/g fresh wt*
17/23				
6:30 PM	−3.57	−2.84	−20.4, −20.8	5.0
8:30 AM	−5.39	−5.44		69.1
10:30 AM	−4.75	−4.97		52.1
1:00 PM	−5.46	−5.09		21.0
8:30 AM[a]	−6.66	−6.13		10.2
22/28				
6:30 PM	−4.77	−4.76	−16.2, −16.0	16.8
8:30 AM	−6.56	−5.88		78.9
10:30 AM	−6.15	−5.14		52.9
1:00 PM	−6.09	−5.09		42.5
8:30 AM[a]	−5.71	−5.51		20.1
27/33				
6:30 PM	−4.93	−5.27	−15.0, −14.1	9.9
8:30 AM	−6.17	−6.71		72.1
10:30 AM	−5.80	−5.21		56.4
1:00 PM	−5.69	−5.14		40.3
8:30 AM[a]	−6.61	−7.17		14.4

[a] CO_2-free atmosphere.

Table III. *Derived $\delta^{13}C$ Values for Respired Carbon*

Species (temp.)	Respired Carbon	
	$\delta^{13}C$	*μmol/g fresh wt*
°C	‰	
K. daigremontiana		
17/23	−11.38	14.6
22/28	−4.82	15.5
27/33	−6.59	12.3
B. tubiflorum		
17/23	−9.98	5.2
22/28	−13.08	3.8
27/33	−8.20	4.5

not shown). The change in whole leaf $\delta^{13}C$ value with temperature reflects increasing proportions of CAM photosynthesis.

A small fraction of the malate synthesized overnight is derived from respiratory CO_2. To measure the quantity and isotopic composition of this material, plants were enclosed in a Lucite box which was freed of CO_2 at the time the lights were turned off and the plants were allowed to refix respiratory CO_2 overnight, after which the leaves were sampled. At 8:30 AM, following this treatment, malate was isolated from these leaves and carbons 1 and 4 were analyzed as described above. The isotopic composition of respiratory CO_2 (Table III) was obtained by correction of the observed δ values for malate left at the end of the previous day.

The isotopic composition of carbon-4 of morning malate for each treatment was corrected for randomization by fumarase, for respiration, and for residual malate as described above under "Theory." The resulting isotopic compositions (Table IV, second column) represent the isotopic composition of carbon which is newly introduced from the atmosphere overnight. The net isotope fractionation associated with nocturnal CO_2 uptake (Table IV, third column) is then given by the difference between this value and the δ value of atmospheric CO_2 (−8‰).

Table IV. *Table of δ Values*

Experimentally measured value for carbon-4 of malate (morning), the derived value for carbon-4 of new malate after all corrections are made (this is the value needed for calculation of the isotope fractionation associated with malate synthesis), and calculated isotope fractionation.

Temperature	Experimental C_4	Derived C_4	Isotope Fractionation[a]
°C		‰	
K. daigremontiana			
17/23	−4.30	−3.94	−4.1
22/28	−6.38	−6.30	−1.7
27/33	−7.40	−8.30	+0.3
B. tubiflorum			
17/23	−5.39	−5.07	−3.9
22/28	−6.56	−8.69	+0.7
27/33	−6.17	−5.10	−3.9

[a] Isotope fractionation = $\delta(CO_2) - \delta$ (malate).

Table V. *Kinetic and Thermodynamic Isotope Fractionations Associated with CO_2 Fixation in CAM Plants*

Step	Isotope Fractionation[a]		Reference
	Kinetic	Equilibrium	
	‰		
Gas diffusion	4.4	0	20, 21
Dissolution	1.1[b]	1.1	16, 21
Liquid diffusion	0.7	0	21
CO_2 hydration	2.0[c]	−9	16
PEP carboxylase	2.0	?	23

[a] A positive value indicates that ^{13}C concentrates in starting material; a negative value indicates that ^{13}C concentrates in the product. [b] The equilibrium value has been measured. The ratio of kinetic fractionations for CO_2 dissolution and CO_2 evolution must be equal to this equilibrium value. [c] P. Paneth and M. H. O'Leary, unpublished data.

In the case of *K. daigremontiana*, the aggregate value of all of the corrections is less than 1‰, as seen previously (22). The trend with temperature that is seen in the raw data is somewhat larger in the derived data. The trend in the *B. tubiflorum* data is less clear. The data from the middle temperature regime appear to be out of line, probably because of errors generated in the separation of carbon-1 from carbon-4. The temperature dependence in the derived data is clearly smaller than that in the *K. daigremontiana* data.

DISCUSSION

Comparison with Theory. The isotope fractionations given in Table IV can be compared with the predictions of various models in order to determine what controls the overall rate of nocturnal CO_2 fixation in CAM plants. Incorporation of atmospheric CO_2 into malate during the night involves a series of steps, all of which may potentially affect the overall isotope fractionation. These steps are: (a) stomatal diffusion, (b) dissolving of CO_2, (c) transport of CO_2 in the liquid phase into the cell, (d) hydration of CO_2, (e) absorption of HCO_3^- by PEP carboxylase and subsequent reduction to malate. The fractionations for all of these steps are known (Table V). As presented here, these are all kinetic (rather than equilibrium) processes. However, some of these steps also have an associated equilibrium isotope fractionation, and these values are also given in Table V.

The overall isotope fractionation for malate synthesis is a combination of these fractionations (10, 20), but it is not simply their sum—the total fractionation depends in a complex way on the relative rates of the various steps in the process. We combine steps b to d in order to consider the following three-step model:

$$CO_2(\text{atmosphere}) \rightleftharpoons CO_2(\text{i})(\text{gas phase}) \rightleftharpoons HCO_3^-(\text{cytoplasm}) \rightarrow \text{malate}$$

All of these steps except the last are to some extent reversible, and the model specifically considers the extent to which this reversion occurs. We assume that liquid-phase diffusion is fast. Rates of CO_2 hydration are known (10).

This model and Table V give rise to the following predictions: If CO_2 uptake is limited by stomatal diffusion (that is, if all steps subsequent to diffusion are relatively fast), then the isotope fractionation[5] will be about +4‰. In the other extreme, if PEP carboxylase is limiting and all preceding steps are fast, then the overall isotope fractionation will be about −7‰. If carboxylation and diffusion are jointly limiting, then the fractionation will be between these two extremes, the exact value depending on the relative rates of the various steps.

The isotope fractionations for both species for all three temperatures regimes fall between the carboxylation-limited extreme and the diffusion-limited extreme. The carboxylation and diffusion rates must be within a factor of two of each other, and CO_2 dissolution, transport, and hydration must be substantially faster than these two. In other words, the internal CO_2 pool partitions roughly equally between loss to the atmosphere and carboxylation. In the low-temperature treatment, the balance among the various steps must be virtually the same in the two species, because the isotope fractionations are the same.

Temperature Effects on Isotope Fractionation. Isotope fractionations for simple chemical and physical processes are essentially temperature-independent within the 10°C temperature range studied in this work. The temperature dependence of the isotope fractionation observed in this study is a result of variations in relative rates of individual steps in the CO_2 fixation process. The temperature dependence of gas-phase diffusion rates is modest: diffusion rates vary with the 1.8 power of the absolute temperature (18). For the temperature range employed here, this gives only a 6% increase in diffusion rate, provided that stomatal aperture is temperature independent. However, stomatal aperture may decrease at higher temperature (11). Although the rate of dissolution of CO_2 in the cell sap is not believed to be limiting, the equilibrium constant for this process changes with temperature: the increase from 17 to 27°C causes a 1.35-fold decrease in the solubility of CO_2 (27). This effect will tend to shift the equilibrium for the second step toward the left. The activities of enzymes increase with increasing temperature. Under saturating conditions at pH 8, the activity of PEP carboxylase from *K. daigremontiana* increases by a factor of 2.3 in this temperature interval (2, 19). For the enzyme from *Bryophyllum fedtschenkoi* the factor is 2.8 (12). Even if *in vivo* conditions are less than totally saturating with regard to both substrates, this temperature factor should be approximately correct. Isotope labeling studies in CAM plants indicate that the activity of carbonic anhydrase increases more rapidly with temperature than does the activity of PEP carboxylase (10). Because carbonic anhydrase activity is at least 40-fold higher than PEP carboxylase activity in *K. daigremontiana* and *B. tubiflorum*, the precise value of the carbonic anhydrase temperature dependence does not enter the calculation.

These results lead to the following first-order prediction for the change in the carbon isotope fractionation between 17 and 27°C: If stomatal aperture is constant, and if the cytoplasmic concentration of PEP does not change, then the isotope fractionation should become more positive by 1‰ as a result of the change in CO_2 solubility and in PEP carboxylase activity.

Experimentally, the isotope fractionation in *K. daigremontiana* is approximately −4‰ in the low temperature treatment, increasing to approximately zero in the high-temperature treatment (Table IV). Qualitatively, this means that CO_2 fixation becomes more diffusion limited and less carboxylation limited as the temperature increases. This direction of change with temperature is consistent with that predicted above, but the magnitude of the change is significantly greater than the 1‰ predicted above.

Two additional effects are probably important. First, it is likely that stomatal closure has occurred to some extent at the higher temperature. This will increase the temperature dependence of the fractionation. The degree of closure must be sufficiently small that it does not affect the total overnight accumulation of malate. Gas exchange studies indicate, however, that the rate of malate accumulation decreases at higher temperatures (15). If stomatal closure is the only additional effect, then the change in isotope fractionation requires that stomatal resistance increase by nearly a factor of three in the 10° interval in *K. daigremontiana*, and it is unlikely that the change would be this large. The more likely possibility is that the activity of enzymes which supply PEP might increase sufficiently such that the steady state PEP supply is increased.

The temperature effects in *B. tubiflorum* are smaller and, for experimental reasons, less clear. The small temperature dependence of the isotope fractionation suggests that neither changes in stomatal aperture nor in PEP supply are significant at the higher temperatures in *B. tubiflorum*.

The Deacidification Process. During deacidification, malate is released from the vacuole and decarboxylated. The change in isotopic composition of remaining malate during deacidification can give information about control of this process. The diffusional release of malate, whether active or passive, will show no more than a very small carbon isotope fractionation (less than 0.5‰; 21). Decarboxylation of malate, on the other hand, is known to show a large fractionation (near 30‰; 8). If decarboxylation is the limiting step in the deacidification, then the overall process will show a large isotope fractionation, and the remaining malate will show large isotopic changes over the course of dea-

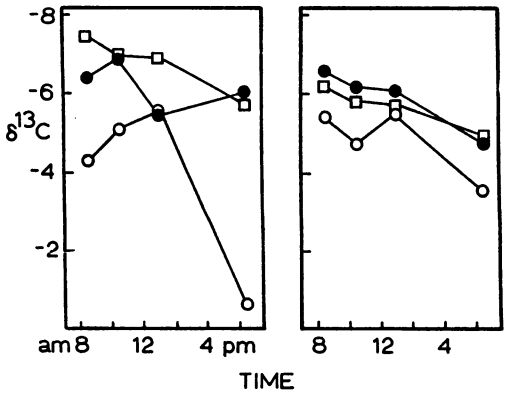

FIG. 1. Isotopic composition *versus* time for carbon-4 of malate from *K. daigremontiana* (left panel) and *B. tubiflorum* (right panel). Temperatures regimes: (○), low; (●), mid; (□), high.

[5] Note that these values are stated as isotope fractionations—that is, as isotopic differences between source CO_2 and product, rather than as absolute isotopic compositions. A positive value for the fractionation indicates discrimination against ^{13}C and in favor of ^{12}C. Thus, these values can be compared with the derived values in the last colum of Table IV.

cidification. On the other hand, if release of malate from the vacuole is the limiting step, then there will be no significant isotope fractionation. Variations in the isotopic composition of carbon-4 of malate for both plants and all three temperature regimes are shown in Figure 1. There is a slight tendency toward more positive δ values (that is, a small isotope fractionation) over the course of deacidification, but the trend is much smaller than that expected if the deacidification process were limited by decarboxylation. Thus, we conclude that deacification is limited principally by release of malate from the vacuole, rather than by decarboxylation.

The one significant exception to these observations is the case of *K. daigremontiana* in the lowest temperature treatment. In this case, the isotopic composition changes by about 5‰ over the course of the deacidification. This change is presumably due to the effect of malic enzyme—at lower temperature the activity of malic enzyme is lower and the overall isotope fractionation reflects a small contribution from the malic enzyme isotope fractionation.

Conclusion. The carbon isotope fractionation associated with malate synthesis in *K. daigremontiana* is temperature dependent. The fractionation is approximately −4‰ at low temperatures and approaches zero at higher temperatures. This change in fractionation is due in part to the temperature dependence of the various components of nocturnal CO_2 fixation: diffusion, dissolution, and carboxylation. In addition, there is also evidence for increased stomatal resistance and increased PEP supply at higher temperatures. The isotopic content of remaining malate over the course of the day indicates that deacidification is controlled by the release of malate from the vacoule, rather than by the activity of malic enzyme.

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