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Short-Term Measurement of Carbon Isotope Fractionation in Plants[1]

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ABSTRACT

Combustion-based studies of the carbon-13 content of plants give only an integrated, long-term value for the isotope fractionation associated with photosynthesis. A method is described here which permits determination of this isotope fractionation in 2 to 3 hours. To accomplish this, the plant is enclosed in a glass chamber, and the quantity and isotopic content of the CO_2 remaining in the atmosphere are monitored during photosynthesis. Isotope fractionation studies by this method give results consistent with what is expected from combustion studies of C_3, C_4, and Crassulacean acid metabolism plants. This method will make possible a variety of new studies of environmental and species effects in carbon isotope fractionation.

Studies begun in the 1950s demonstrated that $\delta^{13}C$ values[2] for plants are more negative than that of atmospheric CO_2; that is, plants contain less ^{13}C than does atmospheric CO_2 (1, 5, 6, 16, 21, 22). Subsequently, it was shown that there is a systematic difference between C_3 plants ($\delta^{13}C$ near −27°/oo) and C_4 plants ($\delta^{13}C$ near −13‰) (2, 3, 24). Within these two broad classes, environmental and species effects are small (16). Delta values for CAM plants are more variable because they reflect the two available photosynthetic modes: Nocturnal CO_2 fixation introduces carbon with a $\delta^{13}C$ value near −11‰, whereas daytime CO_2 fixation introduces carbon with a $\delta^{13}C$ value near −27°/oo (14, 19, 20). Environmental variations which change the CAM/C_3 balance change the $\delta^{13}C$ value accordingly.

Throughout the history of this field, investigators have hoped that isotopic compositions might be useful in studying environmental and species effects. However, except in the case of CAM plants, this has not proved to be the case. Although several thousand species of terrestrial plants have been subjected to isotopic analysis, interspecies variations are small (16). Neither light intensity (21, 26) nor temperature (23, 25–27) produces a substantial change in $\delta^{13}C$. Combustion analysis of 120 strains of *Zea mays* failed to identify any strains which differed significantly from the mean (MH O'Leary, DW Weber, unpublished data). In only a few cases have significant variations been seen: Isotopic compositions of halophytes vary with salinity (10, 15). Isotopic compositions of various strains of wheat correlate with water use efficiency (9). Isotopic compositions of C_4 plants vary slightly with bundle sheath permeability (11).

Measurement of $\delta^{13}C$ values of plant materials has generally been carried out by combustion of dried leaves or other plant parts. The isotopic composition so obtained is a long-term integration of environmental and developmental effects. Early in leaf development, carbon is imported from elsewhere in the plant, and this carbon contributes to the isotopic signal. In a mature leaf, carbon is incorporated as a result of local photosynthesis, but some of this carbon is exported to elsewhere in the plant. As senescence approaches, the export rate increases and the photosynthetic rate decreases. All these phenomena contribute to the isotopic composition obtained in combustion analysis. This long-term integration undoubtedly masks a number of effects and is probably responsible for the lack of environmental and species variations cited above.

A more detailed isotopic signal can probably only be obtained by short-term studies in which the isotopic composition (or fractionation) is measured over a period of a few hours. In the case of CAM plants, we have established a method for studying the isotope fractionation associated with nocturnal CO_2 fixation that makes use of the isotopic composition of carbon-4 of newly formed malate (17). This isotopic information reflects the history of the plant during a short term and is more nearly independent of long-term influences. Studies have been made of the effect of temperature (8), CO_2 concentration (12), and species (17) on the isotope fractionation associated with nocturnal CO_2 fixation. A similar short-term approach to C_3 and C_4 plants should reveal short-term variations in isotope fractionation which are not currently measurable. For a variety of reasons, methods analogous to the malate method are unlikely to be successful. We describe in this paper a general method in which the change in isotopic composition of atmospheric CO_2 is monitored during photosynthesis in a closed compartment. A similar method based on gas-exchange has been used by Berry (JA Berry, personal communication).

MATERIALS AND METHODS

Plants. All plants were grown in the University of Wisconsin Biotron with a 10 h d. The night temperature was 17°C and the day temperature was 23°C. Plants were watered daily with half-strength Hoagland solution. Experiments were conducted 2 to 4 h after the lights were turned on for C_3 and C_4 plants. For CAM plants, nocturnal fixation studies were conducted 2 to 4 h after the lights were turned off, and daytime fixation studies were conducted 6 to 9 h after the lights were turned on.

Soybean (*Glycine max*) was strain Mitchell. The two youngest triplets of fully expanded leaves were used in each experiment. *Zea mays* was strain W64A. The youngest fully expanded leaf was used for isotope fractionation studies. *Kalanchoe daigremontiana* was from the same clone used in previous studies in our

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[2] The definition of $\delta^{13}C$ is

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

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 = $\delta^{13}C_{source} - \delta^{13}C_{product}$.

laboratory (8). The eight youngest fully expanded leaves were used.

Isotope Fractionation Experiment. The reaction chamber consisted of a metal base plate sealed to an 12-L glass bell jar. The plate was divided in half so that the stem of the plant could be introduced through the center. The plate had inlet and outlet ports for pumping air through the chamber. The amount of leaf was chosen so that at least 75% of the initial CO_2 was taken up in about 1 h.

The petiole of the plant in question was sealed with Apiezon Q between two halves of the plate, and then the two halves were clamped together and sealed with wax. The plate was supported on a ring stand. Then the bell jar was placed over the leaves and sealed to the plate with stopcock grease. Air was pumped through the bell jar at a flow rate of 4.5 L/min for 15 min. (it is important that the air intake be placed near the air intake to the room, so that effects of CO_2 from experimenters' breath is minimized). Stopcocks on the entrance and exit ports were closed and the air in the bell jar was sampled by means of an evacuated 1-L flask equipped with a vacuum stopcock. Entrance and exit stopcocks were reopened, and air was flushed through the chamber for a further 15 min, after which the stopcocks were closed again. After a 5-min wait, the air in the bell jar was sampled again by the same procedure. The bell jar was then flushed with air for a further 15 min, and the system was sealed again. After an appropriate wait, a further sample was taken. This flush-wait procedure was repeated as many times as desired in order to accumulate the desired set of time points. The time reported for each point (and shown in the Figures) is the time after sealing. Exposure times were generally at 5 min intervals. For a 40-min exposure time, more than half of the initial CO_2 was invariably gone.

Isotopic Analyses. The CO_2 samples so collected were purified on a high-vacuum line equipped with a diffusion pump. Sample sizes were calculated by use of a calibrated manometer or, for small samples, from the pressure when the sample was admitted to the inlet of the mass spectrometer. Volumes of sample flasks were accurately known. The bell jar system was tight enough that sampling reduced the pressure in the system by approximately one-twelfth and this was taken into account in calculating CO_2 concentration. Final sizes of CO_2 samples were 2 to 15 μmol.

Isotopic compositions were measured on a Finnigan Delta-E isotope-ratio mass spectrometer and were corrected for instrumental effects (7). Repetitive analyses of a single sample could be accomplished with a reproducibility of $\pm 0.05‰$ or better. Delta values are reported relative to the usual PDB standard (7).

Combustions were conducted in sealed, evacuated quartz tubes in the presence of 0.5 g of CuO wire which had previously been heated to 875°C for 2 h. Leaf material was introduced in a Ag boat. After 2 h at 875°C, the temperature was reduced to less than 550°C and the heating was continued for a further 12 h.

THEORY

If a plant is taking up CO_2 in an open atmosphere, then the isotopic difference between leaf and atmosphere corresponds to the isotope fractionation

$$CO_2 \text{ (atm)} \rightarrow \text{leaf}$$
$$\text{isotope fractionation} = \delta^{13}C\ (CO_2) - \delta^{13}C\ (\text{leaf})$$

In the absence of industrial activity the $\delta^{13}C$ value for atmospheric CO_2 is relatively constant at about $-8‰$ (17), so combustion analysis of a leaf can be used to obtain the isotope fractionation.

In a closed container, the atmosphere gradually becomes depleted in CO_2 and a more complex mathematical treatment is necessary. Because of the discrimination against ^{13}C in the photosynthetic process, the atmosphere becomes slightly enriched in ^{13}C. As this happens, the isotopic content of newly introduced carbon also changes, even though the isotope fractionation remains constant. Experiments of this type have long been used in chemical systems, and equations are available which permit calculation of the isotope fractionation from the change in isotopic content of either the source or the product (4).

This same approach can be used for obtaining the isotope fractionation associated with CO_2 uptake in plants, provided that the time- and concentration-dependence of CO_2 uptake are known. At reasonably high light, the CO_2 absorbance rate is roughly proportional to the CO_2 concentration. In addition, CO_2 is given off by plants (respiration) in a process that is probably independent of CO_2 concentration. Taken together, these two processes give, for the CO_2 atmosphere,

$$d(CO_2)/dt = -k_1\ (CO_2) + k_2$$

where (CO_2) is the concentration of CO_2 at any time, k_1 is the first-order rate constant for CO_2 uptake, and k_2 is the zero order rate constant for CO_2 release by respiratory processes. Rate constant k_2 is calculated by use of the fact that at the compensation point, $k_2 = k_1\ (CO_2)$. When appropriate initial conditions are included and the equation is integrated, the result is

$$(CO_2) = ([CO_2]_{\text{initial}} - [CO_2]_{\text{comp}})e^{-k_1 t} + (CO_2)_{\text{comp}}$$

where $(CO_2)_{\text{initial}}$ represents the concentration at the beginning of the experiment and $(CO_2)_{\text{comp}}$ represents the concentration at the compensation point. Separate expressions of this form can be written for $^{12}CO_2$ and $^{13}CO_2$, the difference being that k_1 for the two isotopes differs by the isotope fractionation associated with CO_2 uptake (this is the quantity of interest) and relative values of k_2 reflect the isotopic content of respired carbon.

It should be noted that the way the equations are written specifically includes the possibility that respired CO_2 which is released into the chamber may be refixed. Second, this treatment recognizes that the isotopic composition in the chamber is changing.

Combining separate equations of the type given above permits us to predict the change in $\delta^{13}C$ value of atmospheric CO_2 which occurs during photosynthesis. The variables are

(a) $\delta^{13}C$ of atmospheric CO_2 at the beginning of the experiment
(b) the isotope fractionation associated with CO_2 uptake
(c) the rate of CO_2 uptake
(d) the rate of formation and $\delta^{13}C$ of respired carbon

The experimental data were fitted by first using the measured CO_2 concentration *versus* time to give values for k_1 and k_2. In the case of C_3 plants, special care was taken to obtain an optimum value of k_2 based on the changes in isotopic composition late in the experiment. Once these values were derived, the isotope fractionation associated with k_1 was varied until the best fit to the experimental data was obtained. The isotopic content of respired carbon was assumed to be the same as that of whole leaves. This value has only a minor effect on the derived parameters.

RESULTS

Two triplets of mature leaves from a soybean plant were placed in a closed 12 L container in the light, and the atmosphere was sampled at 5 or 10 min intervals for 45 min. During this period, the CO_2 concentration decreased to about 15% of its initial value and the isotopic composition of the CO_2 in the container showed a significant change (Fig. 1).

During the first part of the experiment, the $\delta^{13}C$ value became quite positive (residual CO_2 became enriched in ^{13}C) as the plant discriminated against ^{13}C during photosynthesis. Late in the

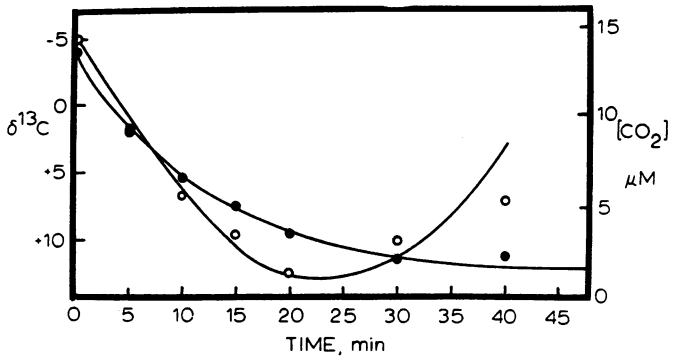

FIG. 1. CO_2 concentration (●) and $\delta^{13}C$ (O) for remaining atmospheric CO_2 *versus* time for CO_2 uptake by soybean at 23°C. Lines represent theoretical values corresponding to an isotope fractionation of 20‰.

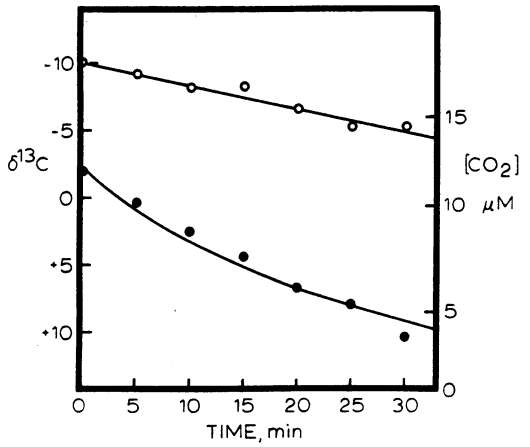

FIG. 2. CO_2 concentration (●) and $\delta^{13}C$ (O) for remaining atmospheric CO_2 *versus* time for CO_2 uptake by maize at 23°C. Lines represent theoretical values corresponding to an isotope fractionation of 4.5‰.

experiment, the rate of photosynthesis became quite small and the isotopic content was principally influenced by respiratory processes. The trend toward more negative $\delta^{13}C$ values late in the experiment was observed consistently. The isotopic composition of respiratory carbon is expected to be much more negative than the atmosphere (perhaps near −27‰; [16]), so the $\delta^{13}C$ value again became more negative. During this period there was little change in CO_2 concentration. Fitting of the isotopic data as described under "Theory" produced an isotope fractionation of 20‰. Atmospheric CO_2 in the Biotron is −8‰, so this isotope fractionation would be expected to produce a leaf $\delta^{13}C$ value of −28‰, if the short-term isotope fractionation is the same as the long-term one. The $\delta^{13}C$ value obtained by combustion of the same leaf was −27.0‰.

Repetition of this same experiment on the next day resulted in the same pattern of CO_2 uptake (data not shown) and gave an isotope fractionation of 20‰. Repetition of the experiment with a different set of leaves under the same environmental conditions resulted in a somewhat different CO_2 absorption rate (on an absolute basis) but a corresponding difference in the pattern of isotopic changes. The scatter in the data was somewhat larger than in the previous experiments, and the calculated isotope fractionation was 17‰.

Similar experiments on a mature leaf of maize showed a similar pattern of CO_2 uptake, except that the CO_2 concentration approached zero at the end of the experiment (Fig. 2; other data not shown). The change in isotopic composition of environmental CO_2 over the course of the experiment was much smaller than with soybean, consistent with the expectation of a smaller isotope fractionation in this case, and there was no tendency of $\delta^{13}C$ values to become more negative late in the experiment. Data fitting by the same procedure gave an isotope fractionation of 4.5‰. If this short-term isotope fractionation is the same as that occurring over the whole life of the leaf, then combustion analysis should produce a $\delta^{13}C$ value of −12.5‰. The observed value was −12.2‰.

CAM plants such as *K. daigremontiana* fix CO_2 at night by a C_4-like pathway and sometimes fix CO_2 in the late afternoon by the direct C_3 pathway (13, 18). The course of the change in isotopic composition during nocturnal CO_2 fixation is similar to that seen in C_4 plants (Fig. 3). The calculated isotope fractionation was 7‰, which would give rise to a whole leaf $\delta^{13}C$ value of −15‰. When *K. daigremontiana* is exposed to CO_2 only during the night, the combustion $\delta^{13}C$ value has been reported to be −11‰ (14). Afternoon CO_2 uptake (3 PM) by the C_3 pathway in *K. daigremontiana* (Fig. 4) gave an isotope fractionation of 19‰, which would give rise to a whole leaf $\delta^{13}C$ value of −26‰, similar to the value of −27‰ seen in *K. daigremontiana* exposed to CO_2 only during the day (14). Evidently these plants are engaging in both CAM (Fig. 3) and daytime (Fig. 4) CO_2 fixation.

DISCUSSION

Qualitatively, the isotopic composition data show the expected pattern: there is a large change in the $\delta^{13}C$ value of remaining CO_2 in the case of C_3 photosynthesis, and only a small change in remaining CO_2 during C_4 photosynthesis. The isotopic pattern of nocturnal CO_2 fixation in the CAM plant *K. daigremontiana* parallels that of C_4 photosynthesis. The isotopic pattern for daytime fixation in the same species parallels C_3 photosynthesis. More quantitatively, the isotope fractionations calculated here are consistent with those expected for C_3 and C_4 photosynthesis, both based on combustion analysis of the leaves actually used in these experiments and on the body of experience regarding combustion-based isotope fractionations for a variety of C_3 and C_4 plants (16).

In general, we do not expect an exact correspondence between the results of the short-term studies and the results of combustion analyses. As noted previously, isotopic compositions obtained by combustion analysis contain information reflecting the entire history of the leaf, whereas the short-term method reflects only a short period. The fact that the short-term method is independent of any isotopic anomalies occurring during development is

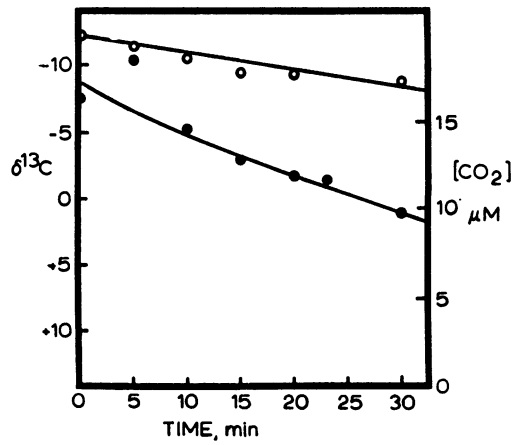

FIG. 3. CO_2 concentration (●) and $\delta^{13}C$ (O) for remaining atmospheric CO_2 *versus* time for CO_2 uptake at night by *K. daigremontiana* at 17°C. Lines represent theoretical values corresponding to an isotope fractionation of 7‰.

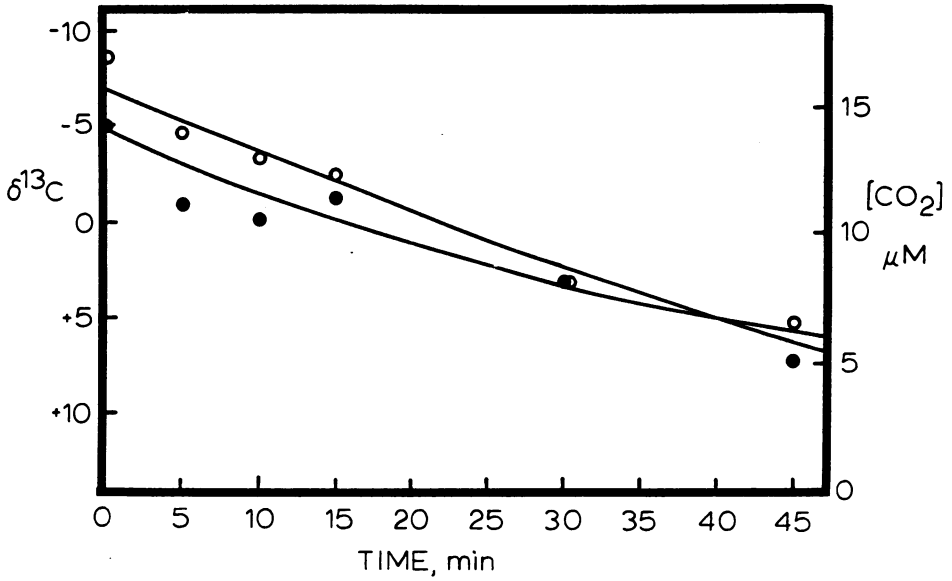

FIG. 4. CO_2 concentration (●) and $\delta^{13}C$ (○) for remaining atmospheric CO_2 *versus* time for afternoon CO_2 uptake by *K. daigremontiana* at 23°C. Lines represent theoretical values corresponding to an isotope fractionation of 19‰.

of particular importance.

Photosynthetic rates respond to CO_2 concentrations, and it is possible that the isotope fractionation might change in the course of these short-term experiments because of changes in stomatal aperture, carboxylation capacity, or other factors. However, the maximum exposure of the leaf to low concentrations of CO_2 is only for a period of about 15 min, and we do not expect that photosynthetic capacity would change enough in that period to perturb the isotopic results. In future experiments, we will collect more data in the early part of the CO_2 uptake curve, thus eliminating this problem.

Respiration is an important confounding variable in studies with C_3 plants. The quantity of respired carbon is small, but its isotopic composition is very different from that of the atmosphere (16). Early in the experiment, the fraction of atmospheric carbon that is derived from respiration is small, but the $\delta^{13}C$ value of that respired carbon is quite differnt from that of the atmospheric CO_2. Later in the experiment, when more CO_2 has been taken up, the proportion of atmospheric carbon that is derived from respiration increases. The mathematical treatment takes account of the fact that some refixation of this respired CO_2 occurs, but both the respiration rate and the isotopic composition of the CO_2 thus produced have an important influence on the derived isotope fractionation. The isotopic compositions late in the experiment (after $\delta^{13}C$ has begun to become more negative) provide a satisfactory basis on which to estimate the respiration correction. In future experiments, the isotopic composition of atmospheric CO_2 will be adjusted to be approximately the same as that of the atmosphere, thus making the respiration correction much smaller.

The method described here for studying isotope fractionation in plants is capable of producing short-term isotope fractionations with a precision of ±1‰, and this can probably be improved in future experiments. Studies of nocturnal CO_2 fixation in CAM plants have shown that short-term experiments provide a wealth of detail that is not available in combustion studies. This method should be particularly useful for study of, *e.g.* developmental effects, environmental effects, and a number of other factors that may affect photosynthetic rates and efficiencies.

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LITERATURE CITED

1. BAERTSCHI P 1953 Die fraktionierung der naturlichen kohlenstoffisotopen im kohlendioxydstoffwechsel gruner pflanzen. Helv Chim Acta 36: 773–781
2. BENDER MM 1968 Mass spectrometric studies of carbon 13 variations in corn and other grasses. Radiocarbon 10: 468–472
3. BENDER MM 1971 Variations in the $^{13}C/^{12}C$ ratios of plants in relation to the pathway of photosynthetic carbon dioxide fixation. Phytochemistry 10: 1239–1244
4. BIGELEISEN J, M WOLFSBERG 1959 Theoretical and experimental aspects of isotope effects in chemical kinetics. Adv Chem Phys 1: 15–76
5. CRAIG H 1953 The geochemistry of the stable carbon isotopes. Geochim Cosmochim Acta 3: 53–92
6. CRAIG H 1954 Carbon 13 in plants and the relationships between carbon 13 and carbon 14 variations in nature. J Geol 62: 115–149
7. CRAIG H 1957 Isotopic standards for carbon and oxygen and correction factors for mass spectrometric analysis of carbon dioxide. Geochim Cosmochim Acta 12: 133–149
8. DELEENS E, I TREICHEL, MH O'LEARY 1985 Temperature dependence of carbon isotope fractionation in CAM plants. Plant Phyisol 79: 202–206
9. FARQUHAR GE, RICHARDS RA 1984 Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust J Plant Physiol 11: 539–552
10. GUY RD, DM REID HR KROUSE 1980 Shifts in carbon isotope ratios of two C_3 halophytes under natural and artificial conditions. Oecologia 44: 241–247
11. HATTERSLEY P 1982 $\delta^{13}C$ Values of C_4 types in grasses. Aust J Plant Physiol 9: 139–154
12. HOLTUM JAM, MH O'LEARY, CB OSMOND 1983 Effect of varying CO_2 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. Plant Physiol 71: 602–609
13. KLUGE M, I TING 1978 Crassulacean Acid Metabolism. Springer-Verlag, New York
14. NALBORCZYK I, LJ LACROIX, RD HILL 1975 Environmental influences on light and dark CO_2 fixation by *Kalanchoë daigremontiana*. Can J Bot 53: 1132–1138
15. NEALES TF, MS FRASER, Z ROKSANDIC 1983 Carbon isotope composition of the halophyte *Disphyma clavellatum* (Haw.)*chinnock* (Aizoaceae), as affected by salinity. Aust J Plant Physiol 10: 437–444
16. O'LEARY MH 1981 Carbon isotope fractionation in plants. Phytochemistry 20: 553–567
17. O'LEARY MH, CB OSMOND 1980 Diffusional contribution to carbon isotope fractionation during dark CO_2 fixation in CAM plats. Plant Physiol 66: 931–934
18. OSMOND CB 1978 Crassulacean acid metabolism: a curiosity in context. Annu Rev Plant Physiol 29: 379–414
19. OSMOND CB, WG ALLAWAY, BG SUTTON, JH TROUGHTON, O QUEIROZ, U LUTTGE, K WINTER 1973 Carbon isotope discrimination in photosynthesis of CAM plants. Nature 246: 5427–5428
20. OSMOND CB, MM BENDER, RH BURRIS 1976 Pathways of CO_2 fixation in the CAM plant *Kalanchoe daigremontiana*. III Correlation with $\delta^{13}C$ value during growth and water stress. Aust J Plant Physiol 3: 787–799
21. PARK R, S EPSTEIN 1960 Carbon isotope fractionation during photosynthesis. Geochim Cosmochim Acta 21: 110–126
22. PARK R, S EPSTEIN 1961 Metabolic fractionation of C^{13} and C^{12} in plants. Plant Physiol 36: 133–138
23. SMITH BN, TW BOUTTON 1981 Environmental influences on $^{13}C/^{12}C$ ratios and C_4 photosynthesis. *In* G Akoyunoglou, ed, Photosynthesis VI. Balaban,

Glenside, PA, pp 255–262
24. SMITH BN, S EPSTEIN 1971 Two categories of $^{13}C/^{12}C$ ratios for higher plants. Plant Physiol 47: 380–384
25. SMITH BN, HMW HERATH, JB CHASE 1973 Effect of growth temperature on carbon isotopic ratios in barley, pea and rape. Plant Cell Physiol 14: 177–182
26. SMITH BN, J OLIVER, C MCMILLIAN 1976 Influence of carbon source, oxygen concentration, light intensity, and temperature on $^{13}C/^{12}C$ ratios in plant tissues. Bot Gaz 137: 99–104
27. TROUGHTON JH, KA CARD 1975 Temperature effects on the carbon-isotope ratio of C_3, C_4, and crassulacean-acid-metabolism (CAM) plants. Planta 123: 185–190