

FINAL REPORT
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Introduction. The objectives of this research are:

1. To develop a theoretical and experimental framework for understanding isotope fractionations in plants.
2. To develop methods for using this isotope fractionation for understanding the dynamics of CO_2 fixation in plants.

Anapleurotic CO_2 Fixation in C_3 Plants (Melzer and O'Leary, 1987, 1991). Although C_3 plants use RuBP carboxylase to fix CO_2 , these plants also contain significant amounts of PEP carboxylase. The role of this latter enzyme in C_3 photosynthesis is unknown. The role of PEP carboxylase in photosynthesis in the C_3 plant Nicotiana tabacum has been probed by measurement of the ^{13}C content of various materials. Whole leaf and purified RuBP carboxylase are within the range expected for C_3 plants. Aspartic acid purified following acid hydrolysis of this RuBP carboxylase is enriched in ^{13}C compared to whole protein. Carbons 1-3 of this aspartic acid are in the normal C_3 range, but carbon-4 (analyzed by treatment of the aspartic acid with aspartate $\text{--} \text{decarboxylase}$) has an isotopic composition in the range expected for products of C_4 photosynthesis (-5 o/oo), and it appears that more than half of the aspartic acid is synthesized by PEP carboxylase using atmospheric $\text{CO}_2/\text{HCO}_3^-$.

In subsequent work, we have extended this study to other species and have studied aspartate derived from protein, free aspartate, malate, and citrate. Enzymatic degradations have been performed in all cases to recover the carbon that might have arisen from CO_2 via PEP carboxylase. The isotopic data from tobacco, spinach, and soybean are similar, and age effects are small. The soybean data are shown in Table I. The isotopic content of whole protein is similar to that of the leaves. Carbon-4 of protein-derived aspartic acid is invariably 15-20 o/oo more positive than the protein, indicating that most of this carbon is derived from action of PEP carboxylase. This presumably happens principally during leaf development, when protein synthesis is rapid and PEP carboxylase concentration is relatively high.

The isotopic content of the pool of free aspartic acid, malic acid, and citric acid indicates that in the mature leaf, these compounds are synthesized principally via the C_3 cycle, although small contributions from PEP carboxylase can be seen. Thus, a principal role of PEP carboxylase in C_3 plants is the synthesis of four-carbon skeletons (principally aspartate) for use in protein synthesis early in development.

Isotope Fractionation in Aspartate Synthesis in Maize. We have used the same approach in maize, in order to determine the isotopic pattern of metabolites in C_4 plants. The results are summarized in Table I. The very positive value observed for carbon-4 of aspartate and malate in these plants is presumably due to the isotope fractionation associated with decarboxylation of four-carbon acids in the bundle sheath cells.

more closed) than at 330 ppm CO₂.

The isotope fractionation decreases as CO₂ concentration increases. This presumably correlates with changing stomatal aperture. Since the leaves are operating near photosynthetic capacity under the conditions of these experiments, stomatal closure enables the plant to maintain internal CO₂ concentration, and thus photosynthetic rate, with decreasing water cost. This is at variance with what we have previously observed in CAM plants.

Interestingly, there is no effect of oxygen concentration on isotope fractionation. The principal effect of reducing oxygen concentration in this range is the virtual abolition of photorespiration. This result indicates that carbon released by photorespiration must have the same $\delta^{13}\text{C}$ value as newly-fixed carbon (i.e., the same as that of the whole leaf).

Table II. Effect of Environmental variables on isotope fractionation in soybean in short-term experiments. Standard conditions: leaf temperature 25 °C, light intensity 450 $\mu\text{Es}/\text{M}^2\text{sec}$, pCO₂ 330 ppm, 60% RH. Isotope fractionations are given as $\delta(\text{source}) - \delta(\text{product})$.

Effect of light intensity:	
150 $\mu\text{Es}/\text{M}^2\text{sec}$	24.5 ‰
450 $\mu\text{Es}/\text{M}^2\text{sec}$	18.4 ‰
Effect of temperature:	
25 °C	18.4 ‰
34 °C	21.0 ‰
Effect of relative humidity:	
at 330 ppm CO ₂ :	
45% RH	17.0 ‰
65% RH	18.4 ‰
at 1010 ppm CO ₂ :	
45% RH	11.0 ‰
65% RH	15.3 ‰
Effect of CO ₂ concentration:	
100 ppm	20.5 ‰
330 ppm	18.4 ‰
1010 ppm	15.3 ‰
Effect of O ₂ concentration:	
2% O ₂	18.0 ‰
21% O ₂	18.4 ‰

Environmental Effects in C₄ Photosynthesis. A series of experiments analogous to those reported in Table II was also carried out with maize. In that case, the variations with conditions are much smaller, generally not exceeding 1 ‰, and they are not statistically significant. This is consistent with the view that C₄ plants are closer to the diffusion-limited extreme and environmental variables have a smaller effect on the P_i/P_a ratio.

Isotopic Consequences of Respiration. We noted above that the carbon released by photorespiration in a C₃ plant must be isotopically the same as the leaf from which it comes. This is the first direct measurement of the isotopic content of carbon released by photorespiration.

Carbon released by dark respiration can be studied in our system by observing the change in concentration and δ¹³C value of the atmospheric CO₂ surrounding the leaf in darkness. The advantage of this method is that it does not require subjecting the leaf to a CO₂-free atmosphere. In the case of soybean, this respired carbon is about 5 ‰ more positive than the leaf. For maize, the respired carbon is 0-1 ‰ more positive than the leaf. These values are similar to the majority of values for respired carbon which have been reported previously. However, it should be noted that there continues to be a significant variation in the isotopic content of dark respired carbon in C₃ plants.

Total respired carbon in the light can also be approached by this method. The limiting CO₂ concentration and δ¹³C value of the atmosphere which is approached at long times can be used to calculate the δ¹³C value of respired carbon (O'Leary et al, 1986).

Developmental Effects in C₄ Plants. Studies of enzyme levels and anatomy in very young C₄ plants have not produced a clear picture of the rate at which the C₄ pathway is expressed during development. Combustion-based isotopic studies are not useful for this purpose because most carbon at this stage is derived from the seed, rather than from photosynthesis. We have used our short-term method to measure the rate of CO₂ uptake and the isotope fractionation associated with this process in maize seedlings at 6, 7, 10, 13, 18, and 22 days of age. Even at 6 days, significant CO₂ uptake occurs. The isotope fractionation is 2.9 to 4.0 ‰ and is independent of age. Thus, even in the youngest plants, the isotope fractionation shows that CO₂ uptake occurs exclusively by the C₄ pathway.

Studies of CAM Plants. Studies by the short-term method of the CAM plant Kalanchoe daigremontiana reveal that the isotope fractionation at night is 2 ‰, as expected for fixation by PEP carboxylase, and consistent with previous studies of malate, whereas the fractionation in the afternoon is 20 ‰, as expected for fixation by RuBP carboxylase. The 'burst' of CO₂ fixation that occurs in the early morning shows a fractionation of 8 ‰, indicating that both pathways are probably operating simultaneously.

Temperature and Light-Intensity Effects on δ¹³C Values. Although thousands of δ¹³C values have been reported, there are few systematic studies of the variation in δ¹³C with

environmental variables such as temperature and light intensity, in spite of the need for such measurements in connection with a variety of environmental studies. We have used the crossed-gradient room in the University of Wisconsin Biotron to grow a variety of C₃ and C₄ plants under varying conditions of light intensity and temperature. Combustion analyses have been performed on mature leaves of a number of these plants.

In the case of C₃ plants, the $\delta^{13}\text{C}$ value becomes more negative by about 3 ‰ as light intensity decreases from 500 to 100 $\mu\text{Es}/\text{M}^2\text{-sec}$. The $\delta^{13}\text{C}$ value also becomes more negative by about 3 ‰ as the temperature increases from 17 °C to 32 °C. Theory predicts that more negative $\delta^{13}\text{C}$ values reflect higher values of P_i/P_a. However, gas exchange studies show no variation in this ratio with light intensity in either C₃ or C₄ plants. We do not know the reason for this discrepancy.

The most important consequence of these results is that past attempts to use $\delta^{13}\text{C}$ values of wood from tree rings as a measure of past climate have all used the wrong value of the temperature effect on isotope fractionation. It is also clear that these climate studies must account for light intensity effects in isotope fractionation--both light intensity effects due to shading and those due to latitude variations.

The temperature and light intensity trend in C₄ plants is much less pronounced than that in C₃ plants. Variations of light intensity and temperature over the same range produce a total change in $\delta^{13}\text{C}$ of no more than 1 ‰ over the entire range of conditions studied. This is consistent with gas-exchange studies and with our studies by the short-term method.

Isotope Fractionation and Water Use Efficiency in Wheat. Recent work from Farquhar's group, building on earlier collaborations with the Wisconsin group, suggests that $\delta^{13}\text{C}$ may be correlated with water use efficiency in C₃ plants. In collaboration with Dr. Henry Nguyen, Texas Tech University, we have been studying the isotopic content of 30 genotypes of wheat of known drought resistance in an attempt to confirm Farquhar's suggestion. These strains have been grown side-by side in field plots in an irrigated treatment and in a dry (water-stressed) treatment. Samples were taken at flowering and at maturity. Triplicate combustion analyses were performed for each set.

Plants grown in the irrigated treatment are consistently 1 ‰ more negative than those from the dry treatment. Qualitatively, this occurs because stomata are more open in the irrigated treatment, leading to the more negative $\delta^{13}\text{C}$ values. At maturity, plants are 1 ‰ more positive than at anthesis. This presumably reflects isotope fractionations associated with respiration during senescence, but there is no apparent relationship to water use.

For a given treatment, the total range of $\delta^{13}\text{C}$ values for all genotypes is about 3 ‰. This is similar to the range of values seen in Farquhar's experiments. However, we find no correlation between $\delta^{13}\text{C}$ and drought resistance either in the dry treatment or in the

irrigated treatment.

This appears to be at variance with Farquhar's work. However, note that we are considering drought resistance, whereas Farquhar is considering water-use efficiency, and the two may not be correlated. This will be examined in future studies. Further, note that our plant are field-grown, whereas those of the Canberra group are glasshouse plants.

Isotope Fractionation and Enzyme Levels in Maize. Isotope fractionations can be a useful technique for studying the dynamics of CO_2 uptake by plants as a consequence of the very different isotope fractionations shown by PEP carboxylase and RuBP carboxylase. Small variations in $\delta^{13}\text{C}$ may reflect variations in carbon fixation pathways. In collaboration with Dr. Hideo Sasakawa and Dr. Tatsuo Sugiyama, Nagoya University, we are interested in the correlation of PEP carboxylase and RuBP carboxylase activities in maize leaf sections.

Enzyme levels and $\delta^{13}\text{C}$ values have been measured for leaf sections of the third and fourth leaves of two-week old maize plants. The levels of both enzymes increase severalfold in going from base to tip, and the $\delta^{13}\text{C}$ values become more negative by about 1.5 ‰. If all else is held constant, we expect that a more negative value is the result of an increase in carboxylation capacity or a decrease in stomatal resistance (i.e., an increase in P_i/P_a). The increased levels of PEP carboxylase in parallel with increasingly negative $\delta^{13}\text{C}$ values indicate that variations in PEP carboxylase are probably responsible for the variations in $\delta^{13}\text{C}$ seen in this case. If the variation in $\delta^{13}\text{C}$ were caused by variations in RuBP carboxylase, we would expect more positive $\delta^{13}\text{C}$ values with increasing RuBP carboxylase concentration, which is the opposite of what is seen.

Carbon Loss During Deacidification in CAM Plants. The nocturnal CO_2 uptake by CAM plants provides a useful model for CO_2 uptake by C_4 plants--the two have similar chemistry, and a number of important experiments can be conducted more easily with CAM plants than with C_4 plants. The carbon isotope fractionation which accompanies nocturnal CO_2 uptake in CAM plants has been measured both by analysis of newly-synthesized malic acid and by analysis of residual CO_2 during CO_2 uptake in a closed chamber. In both cases, newly-fixed carbon has a $\delta^{13}\text{C}$ value near -7 ‰. However, combustion studies reveal that whole leaf carbon is about -11 ‰ when a CAM plant obtains all its CO_2 at night. We were interested in learning the source of this isotopic shift.

Previous gas exchange studies have demonstrated that CAM plants often lose a small amount of CO_2 during the morning deacidification period. By working with CAM plants in closed chambers we have been able to isolate this CO_2 (which often totals 1-5 $\mu\text{mol/g}$ fr. wt) and measure its isotopic content. We find that this released CO_2 has a $\delta^{13}\text{C}$ value of +10 to +20 ‰. By material balance, release of this carbon will cause a net shift in the $\delta^{13}\text{C}$ value of the leaf, and we believe that this release is the principal cause of the shift we have seen.

Ploidy Effects on Carbon Isotopic Composition. Ploidy levels affect a number of physiological and photosynthetic parameters in C₃ plants. In order to determine whether isotopic content was also affected, we determined $\delta^{13}\text{C}$ values for genetically identical diploid, tetraploid, and octaploid strains of alfalfa. Plants were grown side-by-side, and the total range of $\delta^{13}\text{C}$ values is less than 1 o/oo. Thus, in spite of significant morphological and biochemical differences, the ratio P_i/P_a seems to be maintained constant across ploidy levels. We had previously observed a similar lack of difference in C₄ plants.

Isotope Fractionation in Malate Synthesis in Field-Grown CAM Plants. We have previously made extensive studies of the isotope fractionation accompanying nocturnal synthesis of malic acid in growth-chamber grown CAM plants. We were interested in determining whether field-grown plants would show the same isotope fractionation. To this end, we collected samples of *Yucca shidigerii*, *Agave desertii*, and *Opuntia basilaris* at the Deep Canyon field station of the University of California and subjected them to the usual malate analysis. We found that, as in the case of growth-chamber grown plants, the $\delta^{13}\text{C}$ value for newly-fixed carbon in carbon-4 of malate is in the range -4 to -7 o/oo. Thus, we conclude that the field plants and the growth-chamber plants are showing similar characteristics.

Publications:

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