Phytochemistry, Vol. 20, No. 4, pp. 553–567, 1981.
Printed in Great Britain.

0031-9422/81/040553-15 $05.00/0

REVIEW

CARBON ISOTOPE FRACTIONATION IN PLANTS

MARION H. O'LEARY

Departments of Chemistry and Biochemistry, University of Wisconsin, Madison, WI 53706, U.S.A.

(*Revised received* 1 *September* 1980)

Key Word Index—Isotope fractionation; carboxylation; respiration; metabolites; carbon fixation; carbon dioxide; C_3 plants: C_4 plants; CAM plants; isotope discrimination.

Abstract—Plants with the C_3, C_4 and crassulacean acid metabolism (CAM) photosynthetic pathways show characteristically different discriminations against ^{13}C during photosynthesis. For each photosynthetic type, no more than slight variations are observed within or among species. CAM plants show large variations in isotope fractionation with temperature, but other plants do not. Different plant organs, subcellular fractions and metabolites can show widely varying isotopic compositions. The isotopic composition of respired carbon is often different from that of plant carbon, but it is not currently possible to describe this effect in detail. The principal components which will affect the overall isotope discrimination during photosynthesis are diffusion of CO_2, interconversion of CO_2 and HCO_3^-, incorporation of CO_2 by phosphoenolpyruvate carboxylase or ribulose bisphosphate carboxylase, and respiration. The isotope fractionations associated with these processes are summarized. Mathematical models are presented which permit prediction of the overall isotope discrimination in terms of these components. These models also permit a correlation of isotope fractionations with internal CO_2 concentrations. Analysis of existing data in terms of these models reveals that CO_2 incorporation in C_3 plants is limited principally by ribulose bisphosphate carboxylase, but CO_2 diffusion also contributes. In C_4 plants, carbon fixation is principally limited by the rate of CO_2 diffusion into the leaf. There is probably a small fractionation in C_4 plants due to ribulose bisphosphate carboxylase.

INTRODUCTION

Atmospheric carbon dioxide contains about 1.1% of the heavier carbon isotope ^{13}C and 98.9% of the lighter isotope ^{12}C. Plants discriminate against ^{13}C during photosynthesis in ways which reflect plant metabolism and environment [1–8]. The purpose of this review is to summarize the data concerning this discrimination and to provide a chemical and physiological basis for understanding the fractionation in terms of the various components of plant metabolism.

This review is organized as follows:

I. Definition of isotopic composition and isotope fractionation.
II. Review of reported isotopic compositions and fractionations in plants, including effects of metabolic and environmental factors.
III. Theoretical treatment of isotope fractionations, including measured fractionations for component processes and mathematical models for the fractionation.
IV. Interpretation of isotope studies in plants.

Expression of isotopic composition and isotope fractionation

Isotopic compositions are measured by means of a specially designed mass spectrometer equipped with two collectors, two amplifiers and a bridging circuit. The output from the mass spectrometer is an isotope ratio compared to some standard. After minor corrections for instrumental effects and for the presence of ^{17}O in the sample, the ratio is converted to the carbon-13 abundance ratio R [9, 10]:

$$R = {}^{13}CO_2/{}^{12}CO_2. \quad (1)$$

In plant physiology and geochemistry applications this is more commonly expressed as a $\delta^{13}C$ value, in units per mil (‰):

$$\delta^{13}C(‰) = \left[\frac{R\,(\text{sample})}{R\,(\text{standard})} - 1\right] \times 1000. \quad (2)$$

Absolute isotope ratios are troublesome to obtain, and for most purposes it is adequate to give $\delta^{13}C$ values relative to some standard. The standard in general use is PDB (belemnite from the Pee Dee Formation in South Carolina; [1, 9]). The isotope ratio for PDB is $^{13}C/^{12}C = 0.01124$ [9]. Organic matter is invariably depleted in ^{13}C compared to PDB, so $\delta^{13}C$ values of organic materials are negative. A less negative figure means richer in ^{13}C, or 'heavier'. Standards for calibration of isotope-ratio mass spectrometers can be obtained from the U.S. National Bureau of Standards.

In the absence of industrial activity, atmospheric CO_2 has a $\delta^{13}C$ value between -6.4 and $-7.0‰$ [9, 11–13]. However, in industrial areas this value may be significantly more negative because of combustion of coal and petroleum, with $\delta^{13}C$ values near $-30‰$ [14]. Although the $\delta^{13}C$ value for atmospheric CO_2 is believed to have remained approximately constant over geologic time [14], increasing combustion of fossil fuels is causing a shift toward slightly more negative values. The $\delta^{13}C$ value for atmospheric CO_2 decreased by approximately 0.6‰ in the period 1956–1978 [13].

CO_2 in greenhouses and in dense forests may be significantly more negative than $-7‰$ because of the contribution of respired CO_2. Leaves obtained near the ground in dense forests are several per mil more negative than ordinary C_3 plants or leaves from higher elevations in the same forests [14a, 14b].

Isotope discrimination is generally expressed as a difference in the $\delta^{13}C$ value between source and product (1):

$$\text{Discrimination} = \frac{\delta^{13}C(\text{source}) - \delta^{13}C(\text{product})}{1 + \delta^{13}C(\text{source}) \div 1000}. \quad (3)$$

Errors in the literature have often resulted from the failure of investigators to assign the proper algebraic sign to their discrimination values.

Chemists investigating isotope fractionations most often express their results in terms of 'isotope effects', k^{12}/k^{13}, the ratio of rate constants for reactions of the respective isotopic substances. If the source is a sufficiently large reservoir that is not appreciably depleted by product formation, then

$$\text{Isotope effect} = k^{12}/k^{13} = R\ (\text{source})/R\ (\text{product}). \quad (4)$$

Thus the discrimination factor is given by

$$\text{Discrimination} = 1000 \times (1 - k^{13}/k^{12}). \quad (5)$$

In most chemical reactions the lighter isotopic species reacts more rapidly than the heavier isotopic species. When this occurs, the discrimination has a positive value and the isotope effect is greater than unity. Carbon isotope discriminations in enzymatic reactions are commonly in the range 0–20‰, though values as large as 60‰ are occasionally observed [15, 16].

In concluding this section I would like to point out two difficulties with the expression of isotopic compositions and isotope fractionations. As noted, errors in algebraic sign have frequently been made as a result of the unfortunate fact that $\delta^{13}C$ values are almost invariably negative. Second, a number with units of 'per mil' (‰) may be of either of two types: it may be an isotopic composition value (cf. equation 2) relative to some standard (commonly PDB); alternatively, it may be an isotope discrimination (cf. equation 3) reflecting a difference in isotopic composition between source and product.

ISOTOPIC COMPOSITIONS OF PLANTS

Early studies

Early observations concerning carbon isotopic compositions of plant materials were made by Craig [17, 18], Wickman [19] and Baertschi [20]. Craig, in the course of an extensive survey of carbon isotopic compositions of natural materials, observed that most plant materials had a relatively constant $\delta^{13}C$ value near −27‰ [17]. He observed that there seemed to be no important species or geographical effects (interestingly, he reported one grass with a $\delta^{13}C$ value of −12‰, in retrospect clearly a C_4 plant). In a subsequent paper, he discussed his results in more detail and speculated on the probable source of the discrimination. He considered possible contributions from environment, CO_2 diffusion, chemical absorption of CO_2 and respiration [18].

Craig's description of the carbon isotope discrimination was confirmed and extended by Park and Epstein [21], who showed that the key carboxylating enzyme in plants, ribulose bisphosphate carboxylase, discriminates against $^{13}CO_2$. They suggested that the primary cause of the difference in isotopic composition between plants and atmospheric CO_2 is the isotope discrimination by this enzyme.

In an attempt to verify their model, Park and Epstein measured the isotopic composition of 'internal CO_2' obtained by treatment of leaves with acid. However, the origin of the CO_2 evolved in these experiments and the relevance of its isotopic composition to the internal CO_2 pool are both unknown. These experiments do not appear to have been repeated.

Park and Epstein also studied effects of light intensity and CO_2 concentration. They observed that CO_2 respired in the dark is up to 8‰ heavier than whole plant carbon. They subsequently extended these observations and also reported that lipids are enriched in ^{12}C by as much as 8‰ compared to the whole plant [22].

C_3 *and* C_4

The plants studied by Craig [17, 18] and by Park and Epstein [21, 22] were C_3 plants, which incorporate CO_2 from the atmosphere by carboxylation of ribulose bisphosphate. In the mid-1960s, the C_4 pathway was discovered [23, 24]. C_4 plants incorporate CO_2 by the carboxylation of phosphoenolpyruvate. The carboxylation product is transported from the outer layer of photosynthetic cells (mesophyll cells) to the inner layer (the bundle sheath), where decarboxylation and refixation by ribulose bisphosphate carboxylase occur. Isotope studies demonstrated that these plants show less negative $\delta^{13}C$ values than C_3 plants [25–27].

This difference in isotopic composition has become one of the standard methods by which C_4 plants can be distinguished from C_3 plants, and a great variety of plant types have been studied [8, 28–37]. Troughton *et al.* [37] reported a mean $\delta^{13}C$ value for C_4 plants of -13.5 ± 1.5; for C_3 plants the value is -28.1 ± 2.5.

The distinction between C_3 and C_4 pathways of photosynthesis has been particularly useful in studies of C_3/C_4 hybrid *Atriplex* species. These hybrids show a variety of leaf anatomies characteristic of intermediate stages between C_3 and C_4, but the $\delta^{13}C$ values obtained are invariably indicative of the C_3 pathway [38–40].

CAM plants

Succulent plants which exhibit crassulacean acid metabolism (CAM) may either fix atmospheric carbon in the manner of C_3 plants (by use of ribulose bisphosphate carboxylase) or else in a time-separated C_4-like sequence in which phosphoenolpyruvate is carboxylated, then reduced, in the dark, forming malate, which accumulates in the vacuole. In the following light period, this malate is decarboxylated and the CO_2 thus formed is fixed by ribulose bisphosphate carboxylase [41, 42]. Following earlier reports that CAM plants show widely varying carbon isotope ratios [26], several groups nearly simultaneously concluded that the isotopic composition of CAM plants reflects operation of the different carboxylation options [43–46].

Osmond *et al.* [43] correlated isotopic compositions of *Kalanchoe daigremontiana* leaves with results of pulse–chase experiments and concluded that $\delta^{13}C$ values are a useful indicator of photosynthetic pathway, with $\delta^{13}C$ values becoming significantly more positive as the plant shifts from predominantly light to predominantly dark CO_2 fixation. Bender *et al.* [44] surveyed a wide variety of CAM plants and used correlation with environmental variables to reach the same conclusion. Lerman and Queiroz [45] used variable photoperiods with young leaves of *K. blossfeldiana* to manipulate carbon fixation

pathways and observed corresponding changes in $\delta^{13}C$ values. Medina and Troughton [46] observed that for a variety of Bromeliaceae species, $\delta^{13}C$ values near $-13^{\circ}/_{\circ\circ}$ were associated with dark CO_2 fixation and values near $-25^{\circ}/_{\circ\circ}$ were associated with the absence of dark fixation. Similar studies have been performed for *Mesembryanthemum* species [47–49].

Osmond *et al.* [50, 51] studied *K. daigremontiana* grown under various temperature and light regimes. Isotopic compositions showed the expected correlations with gas exchange results; under conditions where carbon fixation occurs principally in the dark, $\delta^{13}C$ values become less negative. The same plant species was studied by Lerman *et al.* [52], who observed that younger leaves are more C_3-like. In the same study were reported isotopic compositions for a soluble fraction (said to be mostly malate), starch and insoluble material. Further fractionation of materials from the same species was reported by Deleens and Garnier-Dardart [53]. The most systematic approach to environmental variables and their effect on $\delta^{13}C$ values in *K. daigremontiana* is that of Osmond *et al.* [54], who measured the time course of $\delta^{13}C$ values for plants grown under various water, light and temperature regimes.

Thus it appears that when CAM plants function strictly in the C_3 mode they have $\delta^{13}C$ values near those of C_3 plants, or $-27^{\circ}/_{\circ\circ}$; a CAM plant engaging in only dark fixation should have a $\delta^{13}C$ value near $-13^{\circ}/_{\circ\circ}$. This view has been further strengthened by Nalborczyk *et al.* [55], who showed that when *K. daigremontiana* plants are furnished with CO_2 only in the light (thus eliminating dark carboxylation) they show $\delta^{13}C$ values of $-26^{\circ}/_{\circ\circ}$. If the same plants are supplied with CO_2 only during the dark period, the $\delta^{13}C$ value is $-11^{\circ}/_{\circ\circ}$.

Isotope fractionations have been used to study the occurrence of the CAM photosynthetic mode in relation to various environmental effects [56–62]. Osmond *et al.* [63] have used $\delta^{13}C$ values to identify CAM species among alpine plants. Winter [64] has used $\delta^{13}C$ values to identify CAM species among Madagascar succulents.

Interspecies variations

The largest factor affecting carbon isotopic compositions is the existence of the C_3, C_4 and CAM photosynthetic options. Other environmental variables can sometimes affect $\delta^{13}C$ values (see below). It is not currently possible to say whether, in the absence of environmental differences, various species of, for example, C_3 plants will show different $\delta^{13}C$ values. Variations in the range of $2–5^{\circ}/_{\circ\circ}$ are still within the realm of possibility.

Intraspecies variations

Lowden [65] failed to find significant differences in $\delta^{13}C$ values for several strains of *Zea mays*. Data of Troughton [35] on several C_3 and C_4 plants suggest that variations of up to $3^{\circ}/_{\circ\circ}$ might be observed for various strains of the same species. No extensive study of this type appears to have been undertaken.

Aquatic plants

The $\delta^{13}C$ values of various aquatic plants are often significantly more positive than those of terrestrial C_3 plants and have sometimes been interpreted as indicating the operation of the C_4 photosynthetic pathway. Recent pulse–chase studies indicate that in most cases the C_3 pathway is operating. Species studied include *Thalassia hemprichii*, *Halophila spinulosa* and *Thalassia testudinum* [66–68]. The small isotope fractionations observed are presumably due to slow diffusion of CO_2 in water. Under these conditions, as with algae under certain conditions [3], the CO_2 supply becomes limiting and the expected C_3 isotope fractionation is not expressed.

Effect of fertilization

The nutritional status of a plant might affect the observed isotope discrimination. For timothy grass (*Phleum pratense* L.), well-nourished plants showed more positive $\delta^{13}C$ values than did plants deficient in nitrogen and/or potassium. The difference was about $2^{\circ}/_{\circ\circ}$ for plants grown under a 13°/7° temperature regime and diminished to less than $1^{\circ}/_{\circ\circ}$ for a 32°/26° regime [69]. Although sodium is a required micronutrient in C_4 plants, no sodium dependency was found in *Kochia childsii* or *Chloris barbata* [69a].

Plant organs

Most measurements of $\delta^{13}C$ values are made on leaves. It is important that investigators specify whether this is the case because there may be some variation in isotopic composition for various organs. In tomato, stems and roots are a few tenths per mil less negative than leaves [21]. Seeds are generally more positive (up to $10^{\circ}/_{\circ\circ}$) than leaves [35, 65]. Potato tubers are about $2^{\circ}/_{\circ\circ}$ more positive than leaves [35]. Epidermal and mesophyll tissues in C_3 plants differ by a few tenths per mil [70].

Effects of temperature

Plants could be useful indicators of past climate if carbon isotope ratios varied in a systematic way with with growth temperature. A number of attempts have been made to correlate $\delta^{13}C$ variations of tree rings with climatic variations, but with only limited success [4]. Troughton [35] reported that $\delta^{13}C$ values become slightly more negative (by up to $2^{\circ}/_{\circ\circ}$) with increasing temperature in several C_3 and C_4 plants. Similar results have been obtained by Smith *et al.* [71, 72] and by Bender and Berge [69]. Other studies have failed to find such an effect [36, 73]. If the temperature effect is real, it must be very small.

CAM plants can show large variations in $\delta^{13}C$ values with temperature as a result of changes in the balance between dark fixation and light fixation. In *K. daigremontiana*, an increase in day temperature from 17 to 31° results in a decrease of about $8^{\circ}/_{\circ\circ}$ in $\delta^{13}C$ values [54].

The temperature variation of isotopic composition in micro-organisms presumably results from the change in CO_2 availability with temperature [74–76]. Isotopic variations in micro-organisms can also reflect different CO_2 fixation options [77]. Other studies of micro-organisms have been summarized by Benedict [3].

Effect of salinity

Smith and Epstein [78] measured $\delta^{13}C$ values for a variety of C_3 and C_4 plants from salt marshes but made no correlation with salinity. Card *et al.* [79] showed no effect of salinity on $\delta^{13}C$ values for *Zea mays*, *Gomphrena globosa*, *Raphanus sativus*, *Triticum aestivum*, *Salicornia virginica* and *Spartina foliosa*. In a more extensive study, Guy *et al.* [80] showed that for the halophytes *Salicornia europaea* subsp. *rubra* and *Puccinellia nuttalliana*, increasing salt concentrations resulted in decreased isotope discrimination both in field samples and in

growth chamber samples. The total range of discriminations was 10‰.

Effect of CO_2 concentration on carbon isotope fractionation

Park and Epstein [21] reported that for tomato the carbon isotope fractionation increases slightly with increasing CO_2 concentrations, but the total range of fractionations reported was only about 2‰.

Carbon isotope fractionations in algae subjected to various CO_2 regimes have been reviewed by Benedict [3]. At low CO_2 levels, blue-green algae show only a very small isotope fractionation [74, 81–83]. At low cell densities and CO_2 concentrations above 0.5%, a maximum fractionation of about 24‰ was obtained [74]. Fractionation was reduced at higher temperatures [74]. The concentration effect is principally a reflection of the availability of CO_2. When the CO_2 level is high the cells have an opportunity to discriminate between ^{12}C and ^{13}C, and a large fractionation is observed. When the CO_2 level is low, growth is largely limited by CO_2 availability and the cells use all available CO_2, independent of its isotopic nature.

Effect of light intensity

Park and Epstein [21] showed less than a 2‰ variation in $\delta^{13}C$ values of tomato plants with light intensity. Smith *et al.* [71] observed seemingly random variations in whole plant $\delta^{13}C$ values with light intensity for C_3 plants. *Acacia farnesiana* values decreased by about 5‰ with increasing light intensity, but *Festuca rubra* showed a 2‰ variation in the other direction.

Solvent deuterium effect

Uphaus and Katz [84] showed that when *Nicotiana tabacum* plants were cultured in water containing increased amounts of deuterium, the carbon isotope fractionation was decreased. The fractionation was approximately linear with deuterium content, being 21‰ at 0% deuterium and 15‰ at 60% deuterium. The plants would not grow above this concentration of deuterium. The authors pointed out that this change in fractionation was most likely a consequence of widespread changes in the cell, and they made no attempt to interpret the results quantitatively.

Replacement of hydrogen by deuterium in cultures of *Chlorella vulgaris* [84] and *C. pyrenoidosa* [81] results in similar decreases in carbon isotope fractionation.

Respired carbon

The isotopic composition of a plant is controlled by the isotopic composition of the CO_2 source, the isotope fractionation accompanying CO_2 incorporation, and the isotopic composition and quantity of the CO_2 lost through respiratory processes. A number of measurements of $\delta^{13}C$ values for respired carbon have been made by trapping the CO_2 released by a plant in a CO_2-free atmosphere and measuring its isotopic composition. It should be noted that not all CO_2 which is formed as a result of respiration and other CO_2-forming processes within the plant is actually released to the environment; some of this CO_2 is refixed. Refixation can fractionate carbon isotopes. Consequently, the isotopic composition measured for 'respired carbon' may differ from that of total carbon formed by respiratory processes.

The isotopic composition of CO_2 released from plants in the dark differs only slightly from that of the whole plant. Thus, CO_2 released by dark respiration in tomato was 2–5‰ more positive than the whole plant [22]. For *Triticum aestivum*, released carbon was 5‰ more positive than the leaf [37]. Carbon dioxide released by seedlings of wheat, radish or pea was about 1‰ more negative than the seedling, although in the case of squash the released CO_2 was 1‰ more positive than the whole plant [85]. For tobacco, the released carbon was 1‰ more positive than the starch pool (which should have nearly the same isotopic composition as the leaves) [85a]. Carbon dioxide released by *Pinus radiata* was 4‰ more negative than the whole plant [37]. In four species of C_4 plants, released carbon was 0–2‰ more negative than the whole plant [37, 85].

There appears to be a greater difference in $\delta^{13}C$ values between whole leaves and carbon released in the light. For *Gossypium hirsutum* and *Triticum aestivum* (both C_3 plants) the released carbon was 10 and 12‰, respectively, more positive than the leaf [37]. For *Paspalum dilatatum* and *Zea mays* (both C_4 plants) the released carbon was 3 and 6‰, respectively, more *negative* than the leaf [37]. The large differences observed in C_3 plants may be due to the occurrence of partial refixation of respired CO_2, leading to a discrimination against ^{13}C and the release of relatively positive carbon to the atmosphere.

The measurement of the isotopic composition of released CO_2 in plants suffers from a kind of botanical 'uncertainty principle'. In order to measure the isotopic composition of released CO_2, it is necessary to subject the plant to a CO_2-free atmosphere for a period of time. However, stomatal opening is subject to control by CO_2 levels, so the extent of refixation of respired CO_2 will be different in the CO_2-free environment than it is in a normal environment. The extent of refixation will also be affected by the fact that there is no atmospheric CO_2 to compete with respired CO_2 for ribulose bisphosphate carboxylase. Thus, we cannot be sure that the isotopic composition of 'respired CO_2' in a CO_2-free environment is the same as would be obtained in a normal atmosphere.

Photorespiration in C_3 plants [85b] probably also affects isotopic compositions. In the case of *Atriplex patula* (a C_3 species) a change from 20% oxygen to 4% oxygen caused the whole plant $\delta^{13}C$ value to become more positive by 2–4‰ [86]. A shift in the *opposite* direction was observed in soybean leaves by Smith *et al.* [71]. No oxygen effect was observed in the C_4 plant *Atriplex rosea* [86].

At least some of the variation in the isotopic composition of respired carbon may be due to whether the source of CO_2 for respiration is carbohydrate or lipid [85, 87]. Plant lipids are significantly more negative than other components (see below).

Although decarboxylations often show significant discriminations against ^{13}C [15], the extent to which this discrimination is expressed during respiration is not known. Values of $\delta^{13}C$ in lichens showed seasonal variations which were suggested to be due to enrichment of ^{12}C during the winter (when carbon is being added through photosynthesis) and depletion of ^{12}C during the summer (when carbon is being lost through respiration) [88].

Few isotopic compositions have been measured for respired carbon in CAM plants. Nalborczyk *et al.* [55]

reported that for *K. daigremontiana* the CO_2 released in the light was "even less negative than that normally obtained for C_4 plants", but no details were given. This observation is not entirely surprising. The amount of CO_2 released under these conditions is probably small compared to the total amount being formed by decarboxylation of malate [55, 89]. The recycling mechanism in CAM plants can allow the internal CO_2 concentration in the light to reach quite high levels [90, 91], presumably with relatively modest CO_2 leakage. Under these conditions the internal CO_2 is principally used to form phosphoglyceric acid under the influence of ribulose bisphosphate carboxylase. The latter enzyme shows a large (20–40‰) discrimination against $^{13}CO_2$ (see below) and as a result the internal CO_2 pool at steady-state may be substantially enriched in $^{13}CO_2$. It was the leakage of this material which was presumably observed by Nalborczyk *et al.* [55].

Isotopic compositions of metabolites

Following the discovery that plants show systematic differences in isotopic composition from the source CO_2, a number of investigators have studied the isotopic compositions of various subfractions of the plant. These studies span anatomical variations (leaf vs stem vs root), metabolic fractions (starch vs lipid, etc.), individual compounds (glutamate vs aspartate vs malate, etc.), and, in a few cases, individual carbon atoms (carboxyl groups of various acids, etc.).

Park and Epstein [21, 22] reported that lipids in tomato are enriched in ^{12}C compared to total plant organic matter. Similar observations have been made for cotton and sorghum [92], marine plankton [93], *K. daigremontiana* [53], potato tuber [94, 95], other plants [22, 96], and micro-organisms [81]. The difference between whole leaf carbon and lipid is often near 5‰, but may be as large as 10‰. This depletion may be caused by the isotope fractionation associated with decarboxylation of pyruvic acid [97].

Other gross differences among metabolites are less striking. Lerman *et al.* [52] conducted a partial separation of components from *K. daigremontiana*. Aqueous extracts (presumed to be mostly malate) fell near −12‰, whereas starch from the same plants was near −15‰, and the insoluble residue was −16 to −18‰. Systematic differences were noted with leaf age. In subsequent work from the same laboratory, Deleens and Garnier-Dardart [53] conducted extensive chromatographic fractionations of material from the same source. As always, the lipid fraction was significantly lighter than other fractions. For a mature leaf the isotopic composition of starch was virtually the same as that of the whole leaf. Cellulose was about 3‰ more negative than the whole leaf. Significantly, the acidic fraction which had accumulated at the end of the dark period, presumed to be mostly malate, was about 1‰ lighter than the CO_2 source. Values for a younger leaf were, in some cases, a few per mil lighter, reflecting a greater contribution of C_3 photosynthesis in the younger leaf. Similar results have been reported for *Kalanchoe blossfeldiana* cv Tom Thumb [98].

O'Leary and Osmond [99] have recently purified malate from *K. daigremontiana* at the end of the light period and at the end of the dark period. The evening sample was about 2‰ lighter than the source CO_2. The morning sample of malate was about 1‰ lighter than the source CO_2. The difference between these results and those of Deleens and Garnier-Dardart [53] may reflect either contributions of other acidic compounds in the latter case or variable contributions of respired carbon in the two studies. In the same study, O'Leary and Osmond [99] observed that malate isolated from *Bryophyllum tubiflorum* had the same isotopic composition as the source CO_2 and was about 5‰ heavier than the whole leaf.

Several constituents of a C_3 plant (cotton) and a C_4 plant (sorghum) were studied by Whelan *et al.* [92]. In both cases the lipids were about 5‰ lighter than the whole leaf. In the case of cotton, the amino acid fraction was 6‰ lighter than the whole leaf. Other metabolites were generally within 2‰ of the whole leaf value. The same study included values for a number of individual metabolites, including aspartate, glutamate, alanine, malate and glucose. Significantly, malate in the C_4 plant was more positive than other metabolites or the whole leaf and only about 2‰ more negative than the source CO_2.

Interpretation of plant isotopic compositions would be most simple if metabolites of a particular type were of similar isotopic compositions. However, this does not appear to be the case. Instead, a variety of studies have suggested that different metabolites of a particular class have quite different isotopic compositions. Degens *et al.* [93] reported that different carbohydrates show different isotopic compositions in marine plankton. Whelan *et al.* [92] showed that glutamate is several per mil different from aspartate in both C_3 and C_4 plants. Malic acid and citric acid differ by about 4‰ in cotton [92]. Crystalline oxalic acid secreted by several cactus species was found to have a $\delta^{13}C$ value within 1‰ of the atmosphere, about 5‰ more positive than plant fibers. Interestingly, oxalic acid from spinach was also significantly more positive than the whole plant [100].

The most thorough and convincing case for large variations among metabolites of a given class comes from the work of Abelson and Hoering [81] on photosynthetic micro-organisms. Individual amino acids were purified and their isotopic compositions were measured. In all cases the amino acids were more negative than the source carbon, but the $\delta^{13}C$ values for individual amino acids varied over a wide range. In the case of *Chlorella pyrenoidosa*, for example, some typical values are glutamic acid −18.7, aspartic acid −6.6, serine −5.7, alanine −10.3, leucine −22.7, tyrosine −19.8. Thus, any approach to isotopic fractionations based on pools of amino acids would be doomed to failure.

However, the situation is even more complicated than this; isotopic compositions are far from uniform even for all of the carbons of a particular amino acid. Abelson and Hoering [81] used enzymatic and chemical methods to decarboxylate their amino acids, thus allowing them to determine the isotopic composition of the carboxyl carbons and then (by difference) all remaining carbons. Large differences were often observed. For example, in *Chlorella pyrenoidosa* the carboxyl carbon of glutamic acid is heavier than the remaining carbons by 12‰; for threonine the difference is 20‰; for lysine it is 23‰.

Similar studies do not appear to have been conducted with higher plants. In the recent work of O'Leary and Osmond [99] carbon-4 of malic acid was found to be 1–4‰ heavier than the other three carbons in material purified from *K. daigremontiana* and *B. tubiflorum*.

Summary of factors affecting $\delta^{13}C$ values

It is very clear that a distinction can be made between C_3 plants and C_4 plants on the basis of their $\delta^{13}C$ values. The operation of the various photosynthetic options in CAM plants can also be clearly distinguished. But how many of the other variables discussed in the preceding sections can give rise to significant variations in $\delta^{13}C$ values?

A caution must first be raised with regard to the experimental data. Most investigators appear to assume that the $\delta^{13}C$ value of their source CO_2 is $-7‰$, but measurements of the source are seldom reported. This assumption may not be correct for plants grown in growth chambers, in greenhouses or at sites close to industrial or urban CO_2 sources. Variations in $\delta^{13}C$ for different plants at a single site, rather than absolute values, provide a more adequate experimental approach.

Further, most literature reports of $\delta^{13}C$ values fail to provide any estimate of error or even any statement of the number of repetitions of a particular measurement. Modern isotope-ratio mass spectrometers are capable of measuring $\delta^{13}C$ values for CO_2 with a reproducibility substantially better than $\pm 0.1‰$. Although under ideal circumstances repeated measurements on plant materials may yield errors in the range of $\pm 0.3‰$ or better, it is likely that errors in most reported values should be considered to be closer to $\pm 1‰$.

On the other hand, it is clear from the preceding discussion that there are small environmental and intrinsic variations in $\delta^{13}C$ values. Carefully controlled experimentation is required for unambiguous delineation of these effects.

Because of the possibility for internal comparison, the variations among various plant fractions and individual metabolites are more clearly established. The difference between lipids and other materials is particularly clear.

THEORETICAL TREATMENT OF CARBON ISOTOPE FRACTIONATION

To date, attempts to explain carbon isotope fractionations in plants have been focused on the carbon-fixing enzymes ribulose bisphosphate carboxylase and phosphoenolpyruvate carboxylase. However, many chemical and some physical processes are capable of fractionating isotopes and it is likely that several of those processes may contribute to the overall fractionation. In this section I give estimates of the magnitudes of the fractionations expected in these various processes. I then describe the models which can be used in conjunction with these fractionations in order to predict actual $\delta^{13}C$ values. The mathematical treatment requires use of isotope effects (cf. equation 4), whereas most plant physiologists are more familiar with isotope discriminations (equation 3) so I will give the fractions in both forms.

Thermodynamics vs kinetic fractions

It is important at the outset to note that there are two kinds of isotope fractionations: thermodynamic and kinetic. Thermodynamic fractionations are isotope fractionations occurring for processes which are at equilibrium. Kinetic fractionations are fractionations occurring because different isotopic species are transformed at different rates. These two types of fractionations are often quite different. For example, the kinetic fractionation associated with the enzymatic conversion of a carboxylic acid into CO_2 and some other material is often near $20‰$ [15], but the thermodynamic fractionation in the same process is only about $3‰$ [101].

Our models for isotope fractionation in plants are based on the cumulative fractionations associated with various individual steps (such as stomatal diffusion, CO_2–HCO_3^- equilibration, carboxylation, etc.). Conversion of material from one state to the next may result in either a kinetic or a thermodynamic fractionation, depending on whether the rate of interconversion of the two states is slow or fast compared to the rates of other steps in the sequence. This distinction is extremely important for the construction of models because thermodynamic fractionations are additive, whereas kinetic ones are not. Treatment of isotope fractionations in processes which are near, but not at, isotopic equilibrium is a key factor in the discussion to follow.

Fortunately, there is a simple relationship between kinetic and thermodynamic isotope fractionations. For a given transformation of the type

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B, \tag{6}$$

the equilibrium constant is defined as

$$K_{eq}^{12} = \frac{[^{12}B]}{[^{12}A]}, \tag{7}$$

in which the superscripts refer to the appropriately substituted isotopic species and brackets indicate concentrations. The equilibrium isotope effect for such a process is given by

$$K_{eq}^{12}/K_{eq}^{13} = \frac{k_1^{12}/k_1^{13}}{k_2^{12}/k_2^{13}}. \tag{8}$$

In terms of discrimination factors, this is very nearly equal to

$$D_{eq} = D_1 - D_2. \tag{9}$$

Thus, if we know the equilibrium isotope effect and the kinetic isotope effect in one direction, we can calculate the kinetic isotope effect in the other direction.

When should we use kinetic fractionations and when should we use equilibrium fractionations? It is permissible to use the equilibrium fractionation *only* when a particular transformation is at equilibrium; that is, the rate of the transformation must be rapid compared to the rates of steps preceding and following it. The situation is exacerbated by the fact that isotopic equilibrium is attained many times more slowly than chemical equilibrium. Thus, when in doubt, it is safer to use a kinetic approach.

^{13}C effects vs ^{14}C effects

Isotope fractionations involving ^{14}C are generally assumed to be twice as large as those involving ^{13}C. For diffusion processes this assumption is correct, but for chemical processes the correct factor is 1.9 [102, 103].

COMPONENT PROCESSES

Isotope fractionations for various components of carbon metabolism in plants are summarized in Table 1 and described in detail in the following sections.

Gas diffusion

Isotope fractionation in gaseous diffusion has been misunderstood frequently in the plant physiology

Table 1. Expected values of isotope fractionations in various components of the CO_2 fixation process

Step	Isotope discrimination (‰)	Isotope effect (k^{12}/k^{13})
Gas-phase diffusion of CO_2	4.4	1.0044
Dissolution of CO_2*	−0.9	0.9991
Liquid-phase diffusion of CO_2 or HCO_3^-	0.0	1.000
CO_2 hydration*	−7.0	0.993
Carboxylation of phosphoenolpyruvate		
Relative to HCO_3^-	2.0	1.002
Relative to CO_2	−5.0	0.995
Carboxylation of ribulose bisphosphate†	30	1.03
Respiratory decarboxylations	0–20	1.00–1.02

* Equilibrium isotope effect.
† Exact magnitude uncertain.

literature. Many authors have concluded that the ratio of diffusion coefficients of $^{12}CO_2$ and $^{13}CO_2$ should be the ratio of the square roots of the masses (thus approximately 11‰), presumably because this relationship holds for the relative diffusion coefficients of water and CO_2 [104, 105]. This relationship, however, is fortuitous, and the correct relationship for gases diffusing in air is the ratio of the square roots of the *reduced masses* of CO_2 (mass 44 or 45) and air (mass 28.8) [105, 106]. This provides an isotope discrimination of 4.4‰ for CO_2 diffusing in air. This factor is independent of temperature and, within broad limits, independent of pressure and CO_2 concentration.

This isotope fractionation will occur both during the diffusion of CO_2 through the boundary layer, through the stomata, and into the internal air spaces of the leaf and during the reverse diffusion of internal CO_2 back to the atmosphere. There is, of course, no equilibrium isotope fractionation in diffusion. To the extent that diffusion is rapid compared to other components of the overall carbon absorption process, no fractionation will be observed.

Dissolution of CO_2

The equilibrium carbon isotope fractionation in the dissolution of CO_2 in water is −0.9‰, with ^{13}C concentrating in the dissolved phase [107]. A proper value for the kinetic fractionation has not been measured, but a logical upper limit for this value is the gas-phase diffusional isotope fractionation of 4.4‰. Isotope fractionations in physical processes are ordinarily quite small. We expect that dissolution of CO_2 will ordinarily be at equilibrium, so the kinetic fractionation should not be important.

Craig [17] and Baertschi [20] measured isotope fractionations near 16‰ for the dissolution of CO_2 in aqueous $Ba(OH)_2$ solution, but Craig [18] correctly pointed out that these fractionations have little to do with the situation in the plant. The experiment was conducted in such a way that the rate-determining step was not dissolution, but rather reaction of CO_2 with OH^-. The latter reaction is relatively slow [108] and should show a large isotope effect. Because plants contain carbonic anhydrase [109], it would not be expected that such a large fractionation in absorption would occur in a plant. A similar phenomenon was observed by Gaastra [110], who attempted to measure the boundary-layer resistance to CO_2 transfer at the interface between air and a leaf replica saturated with KOH solution. His data indicated that CO_2 absorption at the interface was not complete, again presumably because of the relatively slow hydration of CO_2.

Liquid diffusion

Theoretical treatments are currently inadequate for predicting the ratio of diffusion coefficients of $^{12}CO_2$ and $^{13}CO_2$ in aqueous solution, but a variety of experimental and theoretical data indicate that the effect should be no larger than a few tenths per mil ([110]; R. Mills, personal communication).

Hydration of CO_2

The isotope effect on the hydration of CO_2 has been measured a number of times, most recently by Mook *et al.* [107], who presented data covering a range of temperatures. At 25° the isotope fractionation between gaseous CO_2 and dissolved HCO_3^- is −7.9‰. The fractionation decreases slightly with increasing temperature. Note that at equilibrium, ^{13}C concentrates in HCO_3^-. Sign errors involving this fractionation have caused numerous errors in interpretation of isotope fractionations.

No satisfactory estimates are available for the magnitude of the kinetic isotope fractionation associated with the hydration of CO_2. Isotope fractionations in reactions in which CO_2 is formed can be as large as 60‰, and it is possible that such an effect might occur in CO_2 hydration. However, most plants appear to contain carbonic anhydrase in sufficient levels to maintain equilibrium [109]. Under these conditions no kinetic isotope fractionation should be observed.

Phosphoenolpyruvate carboxylase

The initial carboxylation in C_4 plants and in CAM plants during dark fixation is catalysed by phosphoenolpyruvate carboxylase. This enzyme requires HCO_3^- as

substrate [112, 113]. The carbon isotope fractionation associated with carboxylation has been measured several times by combustion methods [114–116]. Schmidt *et al.* [117] measured the isotope fractionation by observing isotopic changes in a limited pool of source CO_2. O'Leary *et al.* (unpublished results) compared the isotopic composition of carbon-4 of the carboxylation product with that of the source HCO_3^-. All three methods indicate that the isotope fractionation is in the range 2.0–2.5‰.

Another way to consider the isotope fractionation in PEP carboxylase is to assume that sufficient carbonic anhydrase is present that the CO_2–HCO_3^- interchange is at isotopic equilibrium. This interchange contributes −7‰ to the isotope fractionation; thus if we use dissolved CO_2 as our reference state in PEP carboxylase, the overall fractionation becomes −5‰. This alternative is often useful, for it saves the necessity of introducing an additional step into the mathematical modelling.

Ribulose bisphosphate carboxylase

Park and Epstein [21] recognized the key role of ribulose bisphosphate carboxylase in carbon isotope fractionation and measured an isotope fractionation of 17‰ compared to the total CO_2–HCO_3^- pool. This corresponds to a fractionation of only 9‰ compared to CO_2. Subsequent measurements have invariably given larger values. Deleens *et al.* [115] reported isotope fractionations in the range 48–99‰ for carboxylase from spinach and maize. The details of these experiments have never been reported. Christeller *et al.* [118] reported a discrimination of 27‰ for the carboxylase from soybean at 25°. The discrimination was nearly independent of temperature.

In one of the most careful studies, Estep *et al.* [119] studied isotope fractionations with purified enzyme and with enzyme from freshly lysed spinach chloroplasts. In both cases the fractionation was 36‰ at 30°. The carboxylase from *Agmenellum quadruplicatum* gave an isotope fractionation of 32‰. Fractionations in the range 28–38‰ were observed for carboxylases from other organisms. Experiments with the spinach enzyme revealed that the fractionation increased to 42‰ when Mn^{2+} was substituted for Mg^{2+}. Substitution of Ni^{2+} reduced the fractionation to 30‰. Estep *et al.* [120] subsequently reported that the carboxylase from *Cylindrotheca* sp. gave a fractionation of 33‰.

Whelan *et al.* [114] reported a carbon isotope fractionation of 34‰. relative to dissolved CO_2 for the enzyme from *Sorghum*. Subsequent studies from the same laboratory [121] presented a careful analysis of the factors likely to affect the fractionation. An isotope fractionation of 27‰ was obtained for the enzyme from cotton at 35°.

All the studies reported above have been performed by combustion methods, in which the key issue is the comparison of the isotopic compositions of the substrates CO_2 and ribulose bisphosphate with that of the product 3-phosphoglyceric acid. A number of factors complicate the analysis of such a system. In the first place, the isotopic difference between starting materials and products is assumed to reflect only the isotopic fractionation associated with the source CO_2. In fact, this is true only if ribulose bisphosphate is quantitatively consumed in the reaction, so that there can be no isotope fractionation connected with the carbons of this substrate. This factor does not appear to have been accounted for in the earlier studies. Further, the isotopic difference between starting materials and products is multiplied by a factor of six (for the six carbon atoms) in order to calculate the isotope fractionation. Obviously, small errors in the results generate large errors in the calculated isotope fractionation. This analysis also assumes that all materials are absolutely pure: otherwise the combustion analyses are in error. Ribulose bisphosphate obtained commercially is quite impure and even when purified, it decomposes rapidly [121a]. Earlier studies failed to provide rigorous criteria of purity for starting materials and products.

Another problem in studies of this isotope fractionation arises from the fact that the substrate for the carboxylation is CO_2, rather than HCO_3^- [122]. In order for the isotopic fractionation measurements to be valid, it is necessary that the interconversion of CO_2 and HCO_3^- be at isotopic equilibrium. Thus, carbonic anhydrase must be present.

Oxygenation also provides a potential source of problems [85b]. If oxygen is not rigorously excluded during the measurements, isotope fractionations can occur because of the carboxylation/oxygenation partitioning. Since oxygenation also produces 3-phosphoglyceric acid, this is a serious problem.

An alternative approach to the isotope fractionation has been taken by Schmidt *et al.* [117], who conducted the carboxylation in the presence of limiting CO_2 and measured the change in isotope ratio of the source CO_2 as the carboxylation proceeded. By this method, enzymes from spinach and maize gave isotope fractionations of 18‰ with a very small temperature dependence.

Thus, ribulose bisphosphate carboxylase shows a large discrimination against $^{13}CO_2$, but the exact magnitude of the discrimination is in doubt. In this author's opinion, the best value is near 30‰, but the uncertainty is near ±10‰.

Finally, one must ask whether the isotope fractionation by ribulose bisphosphate carboxylase must be the same under all conditions. The answer to this question is clearly no. Extensive work of O'Leary and collaborators [15, 16] has shown that the isotope fractionations resulting from enzymatic reactions may vary with pH, temperature, metal ion and other variables. Thus, some of the variation in isotope fractionations found above may reflect real variations with reaction conditions. Fortunately, variation of fractionation with substrate concentration is not generally expected [16].

Respiratory processes

Enzymatic decarboxylations *in vitro* often show significant carbon isotope fractionations [15]. However, based on the isotopic composition of respired carbon, the extent to which these fractionations are expressed *in vivo* appears to be small. Nonetheless, our picture of the isotopic compositions of plants will not be complete until we are able to define accurately the quantity of carbon lost by respiration and the isotopic composition of that carbon. Particularly in C_3 plants, where because of photorespiration the total amount of respired carbon may be large, this is an important consideration.

INTEGRATED MODELS

As noted earlier, fractionations may be of two types, kinetic and thermodynamic. The task for the present section is to provide a general approach for integrating

these fractionation factors to explain the isotope discriminations which might be observed under various conditions. The thermodynamic/kinetic distinction provides a key to this approach.

Most modelling of isotope fractionation to date has been based on the simple assumption of additivity of fractionation factors [2, 3, 35]. This is equivalent to assuming that all steps except the last one in the particular sequence under consideration are at equilibrium; thus, we are dealing with a set of thermodynamic factors plus one final kinetic factor. This model is occasionally correct, but more often it represents an unacceptable simplification.

Another extreme model has sometimes been used. In certain cases an intermediate may be totally sequestered and have no metabolic fate except to be carried through the next step in the pathway. Under these circumstances the isotope fractionation associated with that next step will not be expressed. This occurs, for example, in the decarboxylation–carboxylation sequence in the bundle sheath cells of C_4 plants. Even though the carboxylation of ribulose bisphosphate in the bundle sheath cells should show a large isotope fractionation, this fractionation is not expressed because virtually all of the CO_2 is carried through carboxylation. However, as noted later, the intracellular CO_2 pool which accumulates under such conditions may have a very abnormal isotopic composition.

The dynamics of carbon assimilation processes in plant physiology has most often been considered [104, 123, 124] in terms of a resistivity model in which various metabolic intermediates are connected by resistances which represent the hindrance to metabolic flow through each particular step. The total throughput of the system can then be calculated by analogy with analysis of electrical networks. However, this is not the best approach to computing isotope fluxes because it is necessary when considering isotope fluxes to include specifically the rate of return of material from one metabolic pool to the preceding pool; the resistivity model does not allow this.

The overall modelling of isotope fractionation must be divided into three parts, which will be considered in turn:

1. The initial steps in carbon incorporation. All steps through the *first irreversible step*, most often the carboxylation step, must be included. This approach is sufficient to explain isotopic compositions of whole plants, provided that respiratory processes do not fractionate carbon. Isotopic compositions of individual metabolites or individual carbon atoms cannot properly be understood at this level.
2. Branch points. Isotope fractionations subsequent to the initial steps occur at branch points in metabolic pathways—points at which the intermediate either may suffer two different fates (giving the possibility for different isotope fractionations in the two directions) or else where a very large pool of an intermediate accumulates (allowing for an isotope fractionation from a relatively infinite pool).
3. Non-branching intermediates. Such situations do not actually provide overall isotope fractionation, but it is necessary to discuss such cases in order to achieve a proper view of the isotopic composition of a metabolic intermediate.

I will first derive equations which relate the isotopic composition of photosynthetically-introduced carbon to the isotopic composition of the source, the isotope fractionations in the various steps in the carbon absorption process, and the relative rates of these various steps. If no fractionation of isotopes occurs as a result of either respiratory processes or translocation, then the equations derived here will apply not only to the specific site(s) incorporated during carboxylation, but to the whole leaf as well.

Two-step scheme

Consider a carboxylation system which can be expressed as only two steps. In the first step, atmospheric CO_2 [called CO_2(ext)] diffuses into the plant and becomes internal CO_2 [called CO_2(i)]. This diffusion will to some extent be reversible. In the second step, CO_2 is used to form the first carboxylation product $R{-}CO_2^-$. If carboxylation occurs by way of phosphoenolpyruvate carboxylase, then the second step must include CO_2 hydration. The scheme is summarized as

$$CO_2(\text{ext}) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CO_2(\text{i}) \overset{k_3}{\rightarrow} R{-}CO_2^- . \qquad (10)$$

Transport of material from one state to the next is described by rate constants k_1, k_2 and k_3. For example, the rate of conversion of CO_2(ext) to CO_2(i) is given by the product of k_1 and some power of the concentration of CO_2(i). Under most circumstances it will be adequate to assume that this dependence is on the first power of the concentration. Provided that the power is first or lower, it drops out of the subsequent considerations of isotope fractionation.

Rate constant k_1 describes the diffusion of external CO_2 into the intercellular air space. It thus may include any external boundary layer diffusion, stomatal diffusion, and perhaps an internal diffusion component. Of these, the stomatal resistance to diffusion is generally the most important. This diffusion will show a carbon isotope fractionation of 4.4‰. Rate constant k_2 reflects outward diffusion of CO_2(i) and thus should also show a carbon isotope fractionation of 4.4‰.

The second step (k_3) represents absorption of CO_2 at the air–liquid interface, transport of CO_2 into the cell, equilibration of CO_2 with HCO_3^-, and carboxylation. We assume for the present that absorption and transport are rapid, although direct measurement of the rates of these processes by gas exchange methods has not been possible as yet; most analyses have combined these steps with the carboxylation to give one overall liquid-phase contribution [104, 123]. As noted, the isotope fractionations associated with absorption and liquid-phase diffusion should be extremely small.

In the case of C_4 photosynthesis, hydration of CO_2 to form HCO_3^- must precede carboxylation. As noted previously, we assume that carbonic anhydrase is present in sufficient concentration to maintain this step at equilibrium. Thus, we can logically lump the carboxylation and hydration steps together.

It is implicit in this treatment that the total magnitude of the pool represented by CO_2(ext) is sufficiently large that no change in its concentration or isotopic composition occurs as a result of photosynthetic activity. If this condition is not met, then the apparent isotope fractionation will be smaller than predicted. This was observed, for example, in the work of Berry and Troughton [125] who showed that under conditions where plants absorb all of the CO_2 supplied, no isotope fractionation is observed.

The rate of change of the concentration of CO_2(i) with time is given by

$$\frac{d[CO_2(i)]}{dt} = k_1[CO_2(ext)] - (k_2 + k_3)[CO_2(i)]. \quad (11)$$

Stomatal resistance in higher plants appears to be regulated in such a way as to maintain the concentration of CO_2(i) approximately constant [126]. Certainly at steady-state the change in concentration with time should be very small and it is possible to set equation 11 equal to zero. Thus we find that

$$[CO_2(i)] = \frac{k_1}{k_2 + k_3}[CO_2(ext)]. \quad (12)$$

The item of interest is actually the rate of formation of $R-CO_2^-$, which is given by

$$\frac{d[R-CO_2^-]}{dt} = k_3[CO_2(i)]. \quad (13)$$

(In the general case, we should expect that the rate of formation of the carboxylation product should also depend on the concentration of the other carboxylation substrate, phosphoenolpyruvate or ribulose bisphosphate; however, as we will shortly take the ratio of two expessions of the sort given above, any concentration dependence cancels out.) The internal CO_2 concentration can be substituted from equation 12 into this last expression

$$\frac{d[R-CO_2^-]}{dt} = \frac{k_1 k_3}{k_2 + k_3}[CO_2(ext)]. \quad (14)$$

Although this last expression purports to give the rate of formation of carboxylation products in terms of the concentration of external CO_2, it is unlikely that in this form the equation has any real use. However, the important point is that this equation can provide a proper starting point for explaining the isotope fractionation occurring in these steps. It is possible that the rate constants given above may be different for carbon-12 and for carbon-13. We will call the isotopic rate constants for the first step k_1^{12} and k_1^{13}, respectively and similarly for k_2 and k_3. The ratio k_1^{12}/k_1^{13} is then an 'isotope effect' for that particular step, and we will abbreviate this as

$$E_1 = k_1^{12}/k_1^{13}, \quad (15)$$

and so on for the other isotope effects. Note that these are all kinetic, rather than thermodynamic, isotope effects.

The isotope effect for the whole process, k^{12}/k^{13} (overall), is obtained by writing equations for formation of $R-CO_2^-$ for each isotope species, combining, and integrating

$$\frac{k^{12}}{k^{13}}(\text{overall}) = E_1\frac{E_3/E_2 + k_3/k_2}{1 + k_3/k_2}. \quad (16)$$

Thus, the overall isotope fractionation in this two-step scheme is a composite of four factors: isotope discriminations E_1, E_2, E_3, and the ratio† k_3/k_2.

The values of the isotope effects which would be appropriate for the application of equation 16 to studies of C_3 and C_4 carboxylation are summarized in Table 2. The value of E_3 to be used in connection with C_3 carboxylation is uncertain because of the uncertainty in the correct isotope effect for ribulose bisphosphate carboxylase. No correction for CO_2 hydration is necessary in this case. For C_4 carboxylation, E_3 includes dissolution, CO_2 hydration and carboxylation components.

Values obtained by use of equation 16 can be compared with experimental isotope effects calculated from equation 4 or 5. The remaining unknown is the ratio k_3/k_2.

The ratio k_3/k_2 is a very important quantity. In chemical kinetics it is often called a 'partitioning factor' because it reflects the partitioning of an intermediate [in this case, CO_2(i)] between further reaction and return to the preceding state. When this ratio is large, CO_2(i) mostly reacts further and the carbon fixation rate is limited by diffusion; when the ratio is small, CO_2(i) returns to the starting state much more often than it undergoes carboxylation and the carbon fixation rate is limited by carboxylation. Previous models [3, 21] have failed to take specific account of this partitioning.

It is useful to consider two limiting cases in which equation 16 might be used. If the first step and its reverse are rapid compared to the subsequent carboxylation step,

Table 2. Isotope effects for use in connection with equation 16

Step	Processes included	k^{12}/k^{13}	
		C_3 plants	C_4 plants
1	Gas-phase diffusion into the leaf	1.0044	1.0044
2	Reverse of step 1	1.0044	1.0044
3	CO_2 dissolution and transport, CO_2 hydration (in C_4 plants), carboxylation	1.03*	0.994

* This number is tentative because of the uncertainty in the isotope effect for ribulose bisphosphate carboxylase.

† This rate-constant ratio is for carbon-12, although the ratio for carbon-13 would be only slightly different.

then k_3/k_2 approaches zero and the observed isotope fractionation becomes

$$k^{12}/k^{13}\,(\text{overall}) = E_1E_3/E_2. \tag{17}$$

The fractionation is then the product of the *equilibrium* fractionation in the first step ($= E_1/E_2$) and the kinetic fractionation in the second. This might be the case, for example, when CO_2 diffusion in and out of the leaf is rapid compared to the subsequent carboxylation step. In that case, $E_1 = E_2$ (the isotope fractionation due to diffusion is the same in and out of the leaf) and the observed fractionation becomes equal to the fractionation connected with carboxylation.

The opposite limiting case is the one in which internal CO_2 always undergoes carboxylation and never returns to the atmosphere. In this case k_3/k_2 becomes very large and the observed fractionation becomes equal to E_1, the kinetic fractionation in the first step.

Isotope fractionation and stomatal opening

For the carbon fixation mechanism described by equation 10, we expect that the rate of the first step will be controlled primarily by stomatal resistance, and this will be reflected both in k_1 and in k_2. Later I show that in many plants the partition factor k_3/k_2 is not too different from unity. When that is true, changes in stomatal resistance will result in significant changes in the partition ratio and thus also in the $\delta^{13}C$ value for the plant (cf. equation 16). An increase in stomatal resistance will result in a corresponding *decrease* in rate constants k_1 and k_2 and consequently an increase in k_3/k_2. In C_4 plants an increase in stomatal resistance will cause $\delta^{13}C$ values to become more negative. In C_3 plants an increase in stomatal resistance will cause $\delta^{13}C$ values to become more positive.

Three-step model

If the above two-step model becomes inadequate for describing carbon isotope fractionation (for example, if CO_2 hydration or liquid diffusion must be explicitly considered), it is possible to use the same kind of steady-state assumption (setting expressions of the form of equation 11 equal to zero) for more complex situations. For example, consider the scheme

$$CO_2(\text{ext}) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CO_2(\text{a}) \underset{k_4}{\overset{k_3}{\rightleftharpoons}} CO_2(\text{b}) \overset{k_5}{\rightarrow} R{-}CO_2^-, \tag{18}$$

in which CO_2(a) and CO_2(b) represent two successive states of CO_2 during photosynthesis. In this case the overall isotope fractionation is given by

$$\frac{k^{12}}{k^{13}}(\text{overall}) = E_1\frac{E_3E_5/E_2E_4 + (E_3/E_2)(k_5/k_4) + (k_3/k_2)(k_5/k_4)}{1 + k_5/k_4 + (k_3/k_2)(k_5/k_4)}. \tag{19}$$

Thus the overall fractionation depends on the individual isotope effects E_1 through E_5 and on partitioning factors k_5/k_4 and k_3/k_2.

Fractionations subsequent to carboxylation

The isotopic composition of whole plant carbon is governed by the isotope fractionation which accompanies CO_2 absorption and by any isotope fractionation which accompanies respiration. Even though decarboxylative processes often show significant isotope fractionation [15], such fractionation might not be expressed *in vivo* because no other fate is accessible to the decarboxylation substrate. Consider, for example, a scheme in which an intermediate (I) is converted into some carboxylic acid ($R{-}CO_2^-$) which then undergoes decarboxylation:

$$I \overset{k_1}{\rightarrow} R{-}CO_2^- \overset{k_2}{\rightarrow} R{-}H + CO_2. \tag{20}$$

If the conversion of I to $R{-}CO_2^-$ is irreversible, or nearly so, then any isotope fractionation which should accompany the decarboxylation will not be expressed, and the isotopic composition of the respired carbon will be the same as that of the corresponding carbon atom in I.

It is interesting to note that the isotopic composition of the carboxyl group of $R{-}CO_2^-$ at steady-state may not be the same as that of the intermediate from which it is formed or the CO_2 which is produced. The isotopic composition of the carboxylic acid at steady-state is given by

$$R(R{-}CO_2^-) = (E_2/E_1)(R(I)) \tag{21}$$

where ($R(R{-}CO_2^-)$ and $R(I)$ are the appropriate isotope ratios. Qualitatively, this means that the isotopic composition of an intermediate will adjust itself to compensate for the difference between the isotope effect on its formation and that on its decomposition; the larger the difference in isotope effects, the larger the difference in isotopic compositions. A similar phenomenon holds when any or all of these conversions are reversible.

The important consequence of this fact is that the isotopic compositions of individual metabolites (or, more properly, of particular sites) in plants reflect not only the isotope fractionations in their formation, but also the fractionations in their metabolism. This fact may influence, for example, the isotopic composition of malate isolated from C_4 plants [92]. Malic enzyme shows an isotope fractionation of 30‰ for carbon-4 of malate (M. H. O'Leary and C. Roeske, unpublished results), so the isotopic composition of the malate isolated may or may not reflect the isotope fractionation associated with its formation. This same problem does not occur with malate isolated at the end of the dark period from CAM plants because the malate is not being metabolized under those conditions. The small malate pool remaining at the end of the light period is slightly different from the pool at the end of the dark period [99] because of isotope fractionations connected with the metabolism of malate.

Branch points

As described earlier, a number of cases are known in which individual carbon atoms or individual metabolites have an isotopic composition significantly different from the plant as a whole. This phenomenon often arises as a result of branch points in a metabolic pathway. For example, consider metabolites A, B, C and D in the following scheme.

$$\begin{array}{ccccc} A & \overset{k_1}{\rightarrow} & B & \overset{k_2}{\rightarrow} & C \\ & & \downarrow{\scriptstyle k_3} & & \\ & & D & & \end{array} \tag{22}$$

We assume for the moment that the conversions are all irreversible but the argument is not appreciably different if the conversions are reversible. The isotopic composition of product D compared to that of A ($R(A)/R(D)$) is given

by equation 16. Compounds C and D will have different isotopic compositions if the isotope effects on k_2 and k_3 are different. The operation of this phenomenon in lipid formation has been suggested by DeNiro and Epstein [97].

Calculation of $(CO_2(i))$ *from isotope fractionation measurements*

There is currently a considerable interest in the variation of stomatal resistance in CAM plants in response to CO_2 concentrations [127, 128]. Gas exchange measurements and direct measurements by gas chromatography of $CO_2(i)$ have provided useful information in this regard. Carbon isotope fractionations provide another useful approach.

Consider the model given in equation 10, in which k_1 and k_2 represent inward and outward diffusion of CO_2, and k_3 represents carboxylation. We expect that $k_1 = k_2$; that is, the resistance to CO_2 diffusion is the same in both directions. Equation 12 can be rearranged to give

$$\frac{[CO_2(ext)]}{[CO_2(i)]} = 1 + k_3/k_2. \quad (23)$$

The ratio k_3/k_2 is, of course, obtained from the isotope fractionation measurements, so a direct correlation is expected between gas exchange measurements and isotope fractionation measurements.

CURRENT INTERPRETATION OF ISOTOPE FRACTIONATION IN PLANTS

The purpose of this section is to interpret existing data on isotopic compositions of plants and metabolites in terms of the models given in the preceding sections of this review.

C_3 *plants*

C_3 plants incorporate CO_2 by carboxylation of ribulose bisphosphate. C_3 plants show a large discrimination against ^{13}C, as does ribulose bisphosphate carboxylase, and most authors have assumed that the isotopic composition of C_3 plants simply reflects isotope fractionation due to ribulose bisphosphate carboxylase.

The key question is, what value should we use for the isotope fractionation due to ribulose bisphosphate carboxylase? The range of possible values is large, covering at least 20–40‰. The fractionation of carbon during C_3 photosynthesis is 20‰, and if the fractionation for ribulose bisphosphate carboxylase is also 20‰, then we would say that k_3/k_2 is small, CO_2 diffusion in and out of the leaves is rapid compared to carboxylation, the stomatal resistance is very small, and $CO_2(i)$ should be near 330 ppm.

However, $CO_2(i)$ is smaller than this [126], and the ribulose bisphosphate carboxylase fractionation is probably larger than 20‰. An alternative model is one in which diffusion plays a limited role in reducing the magnitude of the isotope fractionation. If we assume a value of 30‰ for the carboxylase fractionation, then the partitioning factor is 0.6, and stomatal resistance contributes significantly to the overall carboxylation rate. This combination of factors gives a $CO_2(i)$ value of 200 ppm, consistent with what is generally observed for C_3 plants [126]. If this model is correct, then some variation in $\delta^{13}C$ values in C_3 plants might have been expected as a result of variations in the ratio k_3/k_2. However, it is striking that the range of variation of values of k_3/k_2 under a variety of conditions must be quite small. In terms of equation 21, the internal CO_2 concentration in C_3 plants must be maintained nearly constant, independent of environment. This, of course, is consistent with the homeostatic hypothesis [126].

One important and unknown factor complicates the above analysis. We have assumed that respiratory processes make no contribution to the isotopic composition of C_3 plants. Because of the extent of photorespiration in C_3 plants, it would take only a small respiratory fractionation in order to make a significant difference in the overall isotopic composition of the plant. There are differing reports as to whether or not there is isotope fractionation connected with respiration, and this issue must be considered open. The effect of oxygen on $\delta^{13}C$ values in C_3 plants [87] suggests that photorespiration has an important influence on isotopic compositions.

Thus our best guess at this point is that C_3 plants show a large isotope discrimination because of the discrimination in the carboxylation step. However, diffusion is partially rate-limiting and this serves to make the *in vivo* fractionation smaller than the carboxylase fractionation.

C_4 *plants*

The isotope effects given in Table 2 can be used to calculate expected $\delta^{13}C$ values for various diffusion/carboxylation partitionings. If the rate of carbon fixation is entirely limited by carboxylation, then k_3/k_2 (equation 16) is small, k^{12}/k^{13} (overall) = 0.994, and the predicted $\delta^{13}C$ value for C_4 plants is near 0‰. This is, of course, very different from what is observed, and the common assumption that isotope fractionation in C_4 plants simply reflects carboxylation is incorrect.

On the other hand, if diffusion is limiting and carboxylation is relatively fast (k_3/k_2 large), then whole plant carbon should have a $\delta^{13}C$ value near −11‰ in the absence of any respiratory fractionation. Models in which neither diffusion nor carboxylation is entirely limiting give $\delta^{13}C$ values between 0 and −11.

The diffusion limited case is, of course, much closer to what is observed, but it is important to note that the predicted value for the diffusion limited case (−11‰) is slightly different from the accepted C_4 value (−14‰). One mechanism by which this discrepancy could be diminished is isotope fractionation during respiration, but the limited studies available for C_4 plants fail to support this explanation [37].

A more likely possibility is that some leakage of CO_2 occurs during decarboxylation and refixation of CO_2 in the bundle sheath in C_4 plants. Qualitatively, this results in the expression of a small portion of the expected isotope discrimination for ribulose bisphosphate carboxylase; the more CO_2 is lost by this process, the more expression of the ribulose bisphosphate carboxylase fractionation would be observed. Quantitatively, we can use equation 22, in which B is the bundle sheath CO_2 pool, D is CO_2 which has been fixed by ribulose bisphosphate carboxylase, and C is the CO_2 which escapes. Then $E_1 = 1.004$, $E_2 = 1.000$, $E_3 = 1.03$, and a leakage of 5% of the CO_2 pool would shift the predicted $\delta^{13}C$ value from −11 to −13‰. It is unlikely that such a small leakage would have been detected in any experiments performed to date.

Another problem with this model is that it predicts $CO_2(i)$ values approaching zero, whereas experimental values are closer to 100 ppm [126]. This can be

accommodated if diffusion is mostly, but not entirely, limiting in CO_2 absorption and the bundle sheath leakage is somewhat greater than the 5% estimated above.

An important consequence of the conclusion that diffusion primarily limits carbon fixation in C_4 plants is that the photosynthetic rate for C_4 plants appears not to be limited by the amount of phosphoenolpyruvate carboxylase or by the rate of synthesis of phosphoenolpyruvate. Increased efficiency of carbon assimilation in C_4 plants will not be brought about by finding plants with a higher level of carboxylase. Instead, it will be necessary either to increase CO_2 levels or to find a plant with a lower diffusive barrier to CO_2 transport; such a decreased barrier would likely result in a lower water efficiency for the plant.

CAM plants

When CAM plants engage in direct daytime CO_2 fixation, they show isotope fractionations which are identical with those shown by C_3 plants. I have earlier concluded that C_3 plants probably show some diffusional resistance to carbon fixation, and the identical isotope fractionations indicate that CAM plants, too, must show some diffusional resistance to carbon fixation. CAM plants have a much lower density of stomata than C_3 plants [128], but this density does not seem to translate into a greater diffusive resistance to CO_2 transport.

When CAM plants engage in dark CO_2 fixation, their overall isotope fractionation is like that of C_4 plants. However, carbon-4 of malate formed in the dark [99] is significantly more positive than the most positive values which have been observed for CAM plants, indicating that additional fractionation follows the initial carbon fixation. This may either occur as a result of leakage during refixation of CO_2 in the light or else as a result of isotope fractionation during respiration.

Acknowledgements—I am particularly grateful to C. Barry Osmond for advice concerning many aspects of plant physiology. Graham Farquhar suggested the relationship between isotope fractionations and CO_2(i) values. I thank Joe Berry, Margaret Bender, Bruce Smith, Joe Holtum, John Hayes, Klaus Winter and Park Nobel for useful comments on the manuscript. Work in my laboratory was supported by NSF grants PCM77-00812, PCM79-22697 and 2AS-27. This article was written in part during the tenure of a visiting fellowship at the Australian National University.

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