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Anapleurotic CO_2 Fixation by Phosphoenolpyruvate Carboxylase in C_3 Plants[1]

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ABSTRACT

The role of phosphoenolpyruvate 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 ribulose bisphosphate carboxylase are within the range expected for C_3 plants. Aspartic acid purified following acid hydrolysis of this ribulose bisphosphate 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 (obtained by treatment of the aspartic acid with aspartate β-decarboxylase) has an isotopic composition in the range expected for products of C_4 photosynthesis (−5‰), and it appears that more than half of the aspartic acid is synthesized by phosphoenolpyruvate carboxylase using atmospheric CO_2/HCO_3^-. Thus, a primary role of phosphoenolpyruvate carboxylase in C_3 plants appears to be the anapleurotic synthesis of four-carbon acids.

Measurements of the ^{13}C contents of natural materials have been important in understanding the dynamics of CO_2 fixation in plants (12, 22, 23). Initial studies focused on the distinction between C_3 and C_4 plants, but subsequent studies have revealed a wealth of detail with regard to relative rates of carboxylation, stomatal diffusion, CO_2 hydration, and other steps (2, 7, 13).

For isotopic analysis, materials are converted to CO_2 by combustion or chemical or enzymic degradation prior to measurement of the isotope ratio R, defined by

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

For convenience, R values are ordinarily converted to values of $\delta^{13}C$, defined by

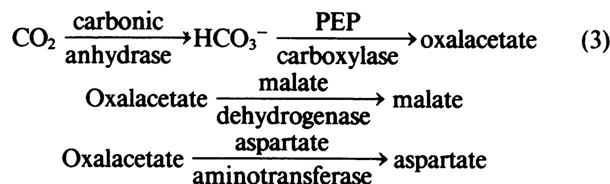
$$\delta^{13}C\ (‰) = (R[\text{sample}]/R[\text{standard}] - 1) \times 1000 \quad (2)$$

The standard ($\delta^{13}C = 0$) is CO_2 obtained from PDB limestone (1). Analysis of whole leaf carbon gives a clear distinction between C_3 plants ($\delta^{13}C$ near −28‰) and C_4 plants ($\delta^{13}C$ near −14%), with virtually no overlap of the two distributions. Theories are now available for explaining why these distributions are different (5, 12, 16). The principal factors are the isotope fractionations associated with stomatal diffusion (4.4‰; Ref. 12), RuBP[2] carboxylase (29‰; Ref. 18), and PEP carboxylase (2‰; Ref. 14).

Combustion-based studies give only an integrated, overall view of carbon metabolism and provide only limited information with regard to specific metabolic pathways. Recently, studies of metabolites have begun to provide more detailed insights into certain pathways. Lipids are unique among metabolites in being significantly depleted in ^{13}C compared to whole leaf carbon (3, 12) and an explanation in terms of the mechanism of fatty acid synthesis has been given (3). The isotopic composition of malate in CAM plants has been used to provide details of environmental effects on nocturnal CO_2 uptake (2, 7, 13).

All plants contain both enzymes of CO_2 fixation, RuBP carboxylase and PEP carboxylase, but the proportions differ with different photosynthetic pathways. In C_4 plants, primary CO_2 uptake is catalyzed by PEP carboxylase, which concentrates CO_2 to improve the efficiency of RuBP carboxylase (4, 8, 17). In this case, the ratio of the two enzymes is near 1:1 (9). C_3 plants, on the other hand, contain a large excess (typically 15:1) of RuBP carboxylase over PEP carboxylase. The role of the latter enzyme in C_3 plants is uncertain. Suggestions have included a role in recapturing respiratory carbon, in synthesizing four-carbon acids (principally malate and aspartate), as well as other possible functions (9).

An attractive possibility for the role of PEP carboxylase in C_3 plants is that it provides four-carbon skeletons for synthesis of aspartate, malate, and perhaps other compounds, as follows.



$$CO_2 \xrightarrow[\text{anhydrase}]{\text{carbonic}} HCO_3^- \xrightarrow[\text{carboxylase}]{\text{PEP}} \text{oxalacetate} \quad (3)$$
$$\text{Oxalacetate} \xrightarrow[\text{dehydrogenase}]{\text{malate}} \text{malate}$$
$$\text{Oxalacetate} \xrightarrow[\text{aminotransferase}]{\text{aspartate}} \text{aspartate}$$

In such a scheme, PEP would presumably be synthesized from products of the C_3 photosynthetic pathway, and only carbon-4 of malate or aspartate would come from the atmosphere via the PEP carboxylase pathway. CO_2 for carbon 4 would be derived from the same CO_2/HCO_3^- pool as is used by RuBP carboxylase (although RuBP carboxylase is in the chloroplast and PEP carboxylase is at least partially in the cytoplasm, there is rapid transport of CO_2 back and forth between the two locations, so it is adequate to consider all CO_2 to be in the same pool).

Even if this scheme is qualitatively correct, we do not know at present what fraction of four-carbon acids might be so derived, nor do we know the extent to which acids derived from this pathway would be in chemical or isotopic equilibrium with products derived from the C_3 pathway. However, insofar as this pathway functions as a source of aspartate or malate, carbon-4 of these materials should be isotopically different from carbons 1–3 because of the difference in isotope fractionations between

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

the C_3 and C_4 photosynthetic pathways. In the limit where all carbon-4 of aspartate is derived via this route and isotope exchange with other metabolites is slow, we would expect carbons 1–3 of aspartate (presumably derived from glycolysis) to be in the usual range for metabolites from C_3 plants (near −28‰; [12, 22, 23]), whereas carbon-4 would be expected to be in the range for metabolites from C_4 plants (near −14‰; [12, 22, 23]), or even more positive (*vide infra*).

Because of the many roles of malic acid in metabolism, and because of the likely randomization of carbon-1 and carbon-4 of malate by fumarase, it is unlikely that an unambiguous isotopic signal can be obtained by studying malate. Instead, we chose to look at aspartic acid, which is kinetically more remote from metabolism (it communicates with malate via aspartate aminotransferase and malate dehydrogenase) and thus has a better chance of preserving an isotopic signal from any contribution of PEP carboxylase. Rather than trying to look at the small and probably dynamic pool of free aspartic acid, we chose to look at a stable form of aspartate, namely aspartate in proteins. To provide a reproducible source of material and to facilitate comparison among species, we focused our attention on the aspartate (and asparagine) in RuBP carboxylase. This enzyme constitutes 25 to 50% of soluble protein in the leaves of C_3 plants and can easily be obtained pure in large quantities (10). We report here that the isotopic content of aspartic acid indicates a significant contribution to aspartic acid synthesis by anapleurotic CO_2 fixation by PEP carboxylase in the C_3 plant, tobacco.

MATERIALS AND METHODS

L-Aspartic acid β-decarboxylase (specific activity 31 units/mg) was from *Alcaligenes faecalis* (19). RuBP carboxylase, purified from leaves of *Nicotiana tabacum*, was provided by Dr. Richard Jensen. Glutamic-oxalacetic transaminase and malate dehydrogenase were obtained from Sigma Chemical Co.

The concentration of aspartic acid was determined by a coupled enzyme method using glutamic-oxalacetic transaminase and malate dehydrogenase (19). Isotope ratio measurements were made on a Finnigan Delta-E isotope-ratio mass spectrometer and are given relative to the usual PDB standard (1). Combustions were conducted in sealed tubes (2). Hydrolysis of protein (40–60 mg per experiment) was carried out with 6 N HCl for 40 min at 155°C. Samples were dried on a rotary evaporator. Yields of aspartic acid were quantitative.

Aspartic acid was separated from acid hydrolysates by ion-exchange chromatography. The concentrated protein hydrolysate was redissolved in 1 ml H_2O and freed from chloride ions by a small column of Dowex 50-X8 (1.1 × 9 cm, 200–400 mesh, H^+ form). After washing with 40 ml of H_2O, the amino acids were eluted with 1 M NH_3 and concentrated to 2 ml. This solution was loaded on an anion exchange column (Bio-Rad AG-1-X8, 1.6 × 30 cm, 100–200 mesh, formate form) and the column was eluted with 250 ml of a gradient from 0.05 to 1 N HCOOH at a flow rate of 50 ml/h. Amino acids were detected with ninhydrin. Aspartic acid eluted as the last amino acid after about 130 ml in a volume of 28 to 32 ml. The combined aspartic acid-containing fractions were brought to dryness on a rotary evaporator. The aspartic acid was redissolved and dried several times to remove traces of HCOOH. Control experiments demonstrated that the aspartic acid so obtained is pure and that aspartic acid subjected to this procedure maintains its isotopic integrity.

Aspartic acid was decarboxylated with aspartate β-decarboxylase (19). To eliminate any isotope fractionation in the decarboxylation, all reactions were carried to 100% decarboxylation. The isotopic fidelity of this procedure has previously been demonstrated (19), and this was confirmed in the present study.

RESULTS

We have obtained pure RuBP carboxylase from *Nicotiana tabacum* and have measured the following isotopic compositions: (a) whole leaf (measured by combustion); (b) pure RuBP carboxylase protein (measured by combustion); (c) pure aspartic acid (*i.e.* carbons 1–4 measured by combustion following acid hydrolysis of the carboxylase and ion-exchange chromatography); (d) carbon-4 of aspartic acid (measured by action of aspartate β-decarboxylase on the aspartate from [c] above); and (e) carbons 1–3 of aspartic acid (by calculation from [c] and [d] above). All measurement techniques have been thoroughly checked. The results of three to five analyses of each type are shown in Figure 1.

The isotopic composition of the whole leaf ($\delta^{13}C = -27.4$‰) is in the range usually observed for C_3 plants (12, 22, 23). The value for RuBP carboxylase protein (−23.0 ± 0.2‰) is slightly more positive. Aspartic acid from RuBP carboxylase (−17.6 ± 0.3‰) is significantly more positive than whole protein, but carbons 1–3 (−21.8 ± 0.4‰) are not significantly different from whole protein. Carbon-4 of aspartic acid (−5.1 ± 0.3‰) is very positive.

DISCUSSION

The isotopic compositions of leaves and of various fractions and metabolites from C_3 plants have isotopic contents averaging near −28‰, with most values falling within ±5‰ of that value. Leaves and various other materials in C_4 plants average near −14‰. Carbon-4 of aspartic acid is at the positive end of the range expected if aspartate is synthesized principally by the action of PEP carboxylase. However, as we will discuss below, because of diffusional and other factors, the isotopic content of C_4 plants does not provide an adequate basis for predicting the isotopic content of carbon-4 of aspartic acid if this material is synthesized by PEP carboxylase in C_3 plants.

Before we conclude that PEP carboxylase is responsible for the abnormal isotopic content of aspartic acid, several other possibilities must be eliminated: (a) There is a large equilibrium isotope effect between aspartate and other metabolites. This is not possible because the equilibrium isotope effect required (more than 15‰) is larger than is reasonable. (b) Aspartate is synthesized via the C_3 pathway with a large kinetic isotope effect. This is not possible because such an isotope effect would tend to deplete aspartate in ^{13}C, rather than enrich it. (c) Aspartate and malate are in isotopic equilibrium and carbon-4 of these materials is becoming enriched in ^{13}C because of the action of malic enzyme. A large isotope effect can be observed for malic enzyme provided that CO_2 is removed as rapidly as it is formed (6). However, in the present case, we would expect the malic enzyme reaction to operate near equilibrium, and the isotope fractionation should be small. Furthermore, if this explanation were correct, then the isotopic composition of carbon-4 of malic acid should be about the same as that of carbon-4 of aspartic acid, and this is not the case (E Melzer, MH O'Leary, unpublished data). Other reactions of malate are not expected to show a large

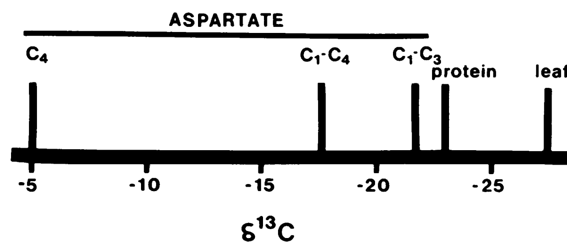

FIG. 1. $\delta^{13}C$ values of various fractions from *Nicotiana tabacum*.

isotope fractionation at carbon-4.

What $\delta^{13}C$ is expected for carbon-4 of aspartate synthesized via PEP carboxylase in a C_3 plant? In C_4 plants, the answer is near −11‰, but in that case stomatal diffusion is principally limiting and the $\delta^{13}C$ value of the internal CO_2 pool is near −11‰. This is not so in C_3 plants. Instead, carboxylation and diffusion are jointly limiting and PEP carboxylase is drawing off only a minor fraction of the same CO_2/HCO_3^- pool used by RuBP carboxylase. The $\delta^{13}C$ value for the internal CO_2 pool in a C_3 plant can be estimated from previous models[3] to be about +1‰. This material is more positive than air because of the isotope fractionation associated with RuBP carboxylase. Synthesis of aspartate involves three steps (a) Conversion of CO_2 to HCO_3^-, which will shift $\delta^{13}C$ to a more positive value[4] by 9‰ (11); (b) absorption of HCO_3^- by PEP carboxylase, which shows an isotope fractionation of 2‰ (14); (c) transamination to aspartate, which should involve no further isotope fractionation. Thus, we estimate that carboxyl carbons so introduced will be about 7‰ more positive than internal CO_2, or near +8‰.

The observed value, −5‰, thus indicates that a major source of aspartic acid in this plant is anapleurotic CO_2 fixation by PEP carboxylase. If the above estimates are correct, then slightly more than half of aspartate is synthesized by this pathway. The remaining aspartic acid is presumably synthesized from products of the C_3 photosynthetic pathway without participation of PEP carboxylase. It has been suggested (9) that PEP carboxylase functions to recapture respired carbon in C_3 plants. Respired CO_2 should have a $\delta^{13}C$ value near that of the leaf and should lead to aspartate having a $\delta^{13}C$ value for carbon-4 of −15 to −20‰. Thus, we cannot eliminate the possibility that some of the remaining aspartic acid is derived via this route, but our data clearly indicate that the primary role of PEP carboxylase is anapleurotic, rather than recapture.

Schmidt and Winkler (21) have discussed the possibility that PEP carboxylase activity has an influence on $\delta^{13}C$ of C_3 plants. Our data suggest that one carbon of one amino acid comes via this route, but for proteins this is probably less than 1% of total carbon. A contribution of this magnitude is consistent with ^{14}C labeling studies of Scheibe and Beck (20). PEP carboxylase might also contribute to the synthesis of other four-carbon acids, but it seems unlikely that this total contribution would exceed 5% of total carbon fixed. The latter value would cause a shift of only 1‰ in the $\delta^{13}C$ value of whole leaf and thus would be a minor contributor to leaf isotopics.

Similar experiments with spinach have revealed a pattern of isotopic labeling consistent with that shown for tobacco above. Experiments are in progress to see whether this same phenomenon occurs in other C_3 plants.

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[3] This is obtained by writing equation 12 of Ref. 12 twice—once for ^{12}C and once for ^{13}C—and taking the ratio of the two equations. The known values of the individual isotope fractionations, the $\delta^{13}C$ value of air (−8‰), and the estimated value for k_3/k_2 (0.6) can then be used to calculate the isotopic composition of internal CO_2.

[4] However, if CO_2 and HCO_3^- fail to reach isotopic equilibrium, this fractionation will be smaller and the resulting oxalacetate will contain more ^{12}C (15). In the limiting case in which HCO_3^- formation is rate limiting, the predicted $\delta^{13}C$ value for carbon-4 of aspartate is the same as that of the internal CO_2 pool, or +1‰. Because of the presence of significant carbonic anhydrase in C_3 plants, the latter case is unlikely.