Fractionation of the Isotopes of Carbon and Hydrogen in Biosynthetic Processes*

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Chapter prepared for a short course sponsored by the Mineralogical Society of America and organized by John W. Valley and David R. Cole. Presented at the National Meeting of the Geological Society of America, Boston, MA, 2-4 November 2001. This pdf can be downloaded from nosams.whoi.edu/jmh/.

This review is concerned with the isotopic relationships between organic compounds produced by a single organism, specifically their enrichments or depletions in $^{13}$C relative to total-biomass carbon. These relationships are biogeochemically significant because

1. An understanding of biosynthetically controlled, between-compound isotopic contrasts is required in order to judge whether plausibly related carbon skeletons found in a natural mixture might come from a single source or instead require multiple sources.

2. An understanding of compound-to-biomass differences must underlie the interpretation of isotopic differences between individual compounds and total organic matter in a natural mixture.

My approach is pedagogic. The coverage is meant to be thorough, but the emphases and presentation have been chosen for readers approaching this subject as students rather than as research specialists. In common with the geochemists in my classes, many readers of this paper may not be very familiar with biochemistry and microbiology. I have not tried to explain every concept from those subjects and I have not inserted references for points that appear in standard texts in biochemistry or microbiology. Among such books, I particularly recommend the biochemistry text by Garrett and Grisham (1999) and the microbiology text by Madigan et al. (2000). The biochemistry text edited by Zabay (1998) is also particularly elegant and detailed. White (1999) has written a superb but condensed text on the physiology and biochemistry of prokaryotes.

A schematic overview of the relevant processes is shown in Figure 1. Plants and other autotrophs fix CO$_2$ to form initial products (C$_2$–C$_6$ acids and carbohydrates). These involve oxidation, reduction, bond formation, and bond cleavage. Some of these processes produce energy and others consume it. They are balanced against each other, and the organism will remain viable as long as net yields of energy are adequate for maintenance. Growth – the production of additional biomass – will be possible if photosyntheate or metabolites remain after adequate quantities of energy have been produced. This will depend not only on the magnitude of the supply but also on the range of ener-

* Dedicated to my father and mother on his 90th birthday and their 65th wedding anniversary, 26 March 2001. Of all the good fortunes that I have enjoyed, none equals that of being his son and their child.
Isotopes in Reaction Networks

Isotope effects and mass balance

An arbitrary sequence of reactions is indicated schematically in Figure 2. Analysis of this system will provide a useful introduction to studies of real organisms. Reactant A, for example, represent a particular carbon position in a two- or three-carbon product of metabolism or photosynthesis. If there were no isotope effect associated with reaction 1 (ε1 = 0), then the isotopic composition of the carbon being transmitted by reaction 1 would be equal to that of reactant A. This follows from a general relationship \( \delta' = \delta - \epsilon \) which can be written as

\[
\delta' = \delta_0' - \epsilon
\]

where \( \delta_0' \) and \( \delta' \) are the instantaneous isotopic compositions of a product and reactant and \( \epsilon \) (expressed in ‰) is the isotope effect associated with the reaction linking R and P. Here we have \( \delta_0' = \delta_A \cdot \epsilon_{\text{A}} \) and, from \( \epsilon_{\text{A}} = 0 \), \( \delta_0' = \delta_A \). To provide a quantitative basis for downstream δ values, we will define \( \delta_A = 0 \) ‰.

1 In order to focus on the principles, we will adopt the approximation represented by equation 1. The precise relationship, which should be used in order to avoid errors in practical work, is given by \( \epsilon = 10^4(\alpha - 1) \), where \( \alpha = [(\delta_0' + 1000)/(\delta' + 1000)] \).

The example depicted in Figure 2 indicates that B is an intermediate between A and C. There is no division of the carbon flow, but there is a significant isotope effect associated with reaction 2, which transforms B to yield C. Two requirements then seem to collide. From equation 1, we know that \( \delta_1 = \delta_2 - \epsilon_2 \). From conservation of mass we have \( \delta_1 = \delta_1 \). Since \( \epsilon_2 = 25\% \), this requires \( \delta_2 = 25\% \) even though \( d_2 \) (the \( d \) value of the carbon flowing to B) = 0 ‰. At steady state, this must be true. Initially, it cannot be. The resolution of these requirements is shown in Figure 3, which depicts isotopic compositions as a function of time. Initially \( \delta_1 = 0 \) and \( \delta_2 = -25\% \). A result of this imbalance, \( \delta_2 \) rises until \( \delta_2 = \delta_1 \). The time constant for an adjustment of this kind is given by \( \tau = m/\phi \), where \( m \) represents the quantity of the intermediate (B in this case) present at steady state and \( \phi \) is the flux (e.g., moles/time) of material through the intermediate pool. Whenever standing stocks are small relative to throughputs, steady-state conditions must be dominant in biosynthetic-reaction networks.

The flow of carbon divides at C, which lies at branch point in the network. From equation 1 and the isotope effects specified for reactions 3 and 4, we have \( \delta_3 = \delta_3 - 15\% \) and \( \delta_4 = \delta_4 - 35\% \). Mass balance requires also that the total amounts of \(^{13}\text{C}\) and \(^{12}\text{C}\) flowing to and from C are equal. In mathematical terms, the mass balance for total carbon is specified by

\[
\varphi_2 = \varphi_3 + \varphi_4
\]

The mass balance for carbon-13 is specified by

\[
\varphi_2 \delta_2 = \varphi_3 \delta_3 + \varphi_4 \delta_4
\]
As shown by the graph, values of \( f_3 \), a parameter equivalent to the fractional composition of the carbon flowing to the left of the graph, indicates that the isotopic composition of carbon flowing to the left of the graph indicates

\[ f_3 = \frac{1}{\phi_3} \delta_3 \]

Isotopic shifts imposed on \( E \) propagate downstream. As a result, the isotopic compositions of \( E, F, G, H, \) and \( I \) are all affected by \( f_3 \). Any investigator seeking to understand the isotopic composition of \( I \), and perhaps focusing his or her attention on reactions 1, 2, 4, 5, 7, and 8 (the pathway linking \( A \) and \( I \)), would have to remember also that the yield of \( D \), a byproduct in this reaction network, could have an important effect on \( \delta_I \).

The appearance of graphs like Figure 4 is controlled largely by the relative magnitudes of the isotopic effects. The lines representing the isotopic compositions of the branching flows will be separated vertically by the difference between the isotopic effects. If \( f \) expresses the fraction of the depleted product, the lines will have a positive slope. The slope will be negative if \( f \) expresses the fraction of the enriched product (compare Figures 4 and 5).

Substances \( E \) and \( F \) are in equilibrium. As a result, their isotopic compositions will vary together, the difference being controlled by the isotope effect associated with the equilibrium. In the example depicted by Figure 2, \( F \) will always be enriched in \(^{13}\text{C} \) by 8‰ relative to \( C \). In this case, the absence of an isotope effect at reaction 7 means that the carbon flowing to \( H \) will have an isotopic composition equal to that of \( F \), but the presence of a branching pathway with an unequal isotope effect (\( \epsilon_6 \neq \epsilon_7 \)) means that significant fractionation can still occur. The resulting isotopic relationships are summarized graphically in Figure 5 and show that \( G \) provides a second example of a byproduct whose yield can influence the isotopic composition of \( I \).

A substantial isotope effect is associated with reaction 8, but the absence of a branching pathway means that the isotopic composition of \( I \) is effectively controlled by \( \delta_3 (\delta_4 = \delta_5) \). The only effect of \( \delta_3 > 0 \) is to require that, at steady state, \( H \) is enriched relative to \( I \).

**Carbon positions vs. whole molecules**

Important words appear in the caption to Figure 2: “Letters indicate carbon positions within reactants and products.”

For example, \( C \) could represent the \( C-2 \) (carbonyl) position in pyruvate. \( D \) and \( E \) would then represent the fates of that position in products derived from the pyruvate. Reactions occur between molecules but isotopic selectivity is expressed as chemical bonds are made or broken at particular carbon positions. Isotope effects pertain to those specific processes and control fractionations only at the reaction site, not throughout the molecule. To calculate changes in the isotopic compositions of whole molecules, we must first calculate the change at the reaction site, then make allowance for the rest of the molecule. In doing so, we inevitably find that the site-specific isotope shift is diluted by admixture of the carbon that was just along for the ride. It follows that the isotopic differences that can be observed between molecules “must be related to, indeed, must be the attenuated and superficial manifestations of, isotopic differences within molecules” (Monson and Hayes, 1982a).

**Useful lessons**

Generalities can be drawn from the foregoing discussion. First, isotopic compositions of intermediates (e. g., \( B \)) can differ substantially from those of final products (e. g., \( I \)). Second, the division of carbon flows at branch points can strongly affect isotopic compositions downstream. As a result, it is practically impossible to predict the isotopic compositions of final, biosynthetic products on the basis of observed isotopic compositions of intermediates. In plain words, if you want to know about the isotopic composition of lipids, it’s very risky to rely entirely on analyses of acetate, even though most lipids are produced from acetate.

On the other hand, if you want to know, for example, why lipids are depleted in \(^{13}\text{C} \) relative to other biosynthetic products, it will be necessary to examine evidence capable of revealing the structure and characteristics of the related network of reactions. Two complementary approaches have been developed thus far. Most straightforwardly, DeNiro and Epstein (1977) devised experiments for the
determination of isotope effects at key points in the reaction network that links \( \text{n-alkyl lipids to metabolites derived from carbohydrates and from some amino acids. In contrast, Abelson and Hoering (1961) pioneered the examination of intramolecular patterns of isotopic order. They studied the biosynthesis of amino acids, analyzing only the end products. However, they determined not only the \( \delta \) values of the individual molecules but also (to the extent possible) the distributions of \( ^{13}\text{C} \) \emph{within} the molecules. A similar approach was later used by Monson and Hayes (1982a) to study isotopic fractionations in lipid biosynthesis.

These reaction-based and product-based lines of investigation are complementary because neither can be perfect or complete and because inferences drawn from their results must be consistent. Even if it were possible to determine the isotope effect at each carbon position for every single step in a reaction sequence, and even if it were possible to recognize in advance every significant feature of the network, it would never be possible to be sure that studying the reactions in isolation was a reliable guide to their characteristics \textit{in vivo}. On the other hand, even if it were possible to determine the isotopic composition at every single carbon position within all the related products of a network, it would never be possible to assign each observed variation reliably to causes that might lie many steps upstream in the sequence of reactions. More positively, results of intramolecular isotopic analyses could show that reaction-based investigations must have overlooked a key step. And, if multiple reaction steps have significantly influenced isotopic compositions in end products, quantitative interpretation of intramolecular patterns of isotopic order requires information developed from reaction-based studies. Examples will be provided in subsequent sections of this review.

\textbf{Further General Factors Affecting Isotopic Compositions}

\textit{Compartmentalization}

A cell is not a stirred reaction vessel. In eukaryotic algae, for example, \( \text{CO}_2 \) is fixed and initial carbohydrates are produced in the chloroplast (also called plastid). Fatty acids are produced in the chloroplast and then exported for use elsewhere in the cell (Ohlrogge and Jaworski, 1997) and all of the \( \text{C}_{20} \) and \( \text{C}_{40} \) isoprenoid carbon skeletons required by the photosynthetic apparatus are plastidic products (Kleinig, 1989). In contrast, sterols, which are derived from a \( \text{C}_{30} \) isoprenoid carbon skeleton, are produced in the cytosol (Lichtenhaler, 1999). In higher plants, the carbon feedstocks required to support biosynthesis outside the chloroplast are exported mainly as the \( \text{C}_3 \) carbohydrate derivative, dihydroxyacetone phosphate (Schleucher \emph{et al}., 1998). As a result of these factors, plastidic fatty acids and cytosolic sterols, both derived from acetate, can have different isotopic starting points. Moreover, even when starting points and downstream processes are closely similar, the separation of pathways between compartments can mean that divisions of carbon flows at branch points differ significantly and, therefore, that final isotopic compositions differ sharply.

Smaller and larger organisms can present correspondingly simpler and more complex isotopic relationships. Prokaryotic cells, those of Archaea and Bacteria, are much smaller and lack the internal boundaries that stabilize chemical compartmentalization in Eukarya. As a result, compounds with similar structures and deriving from the same biosynthetic pathway \textit{usually} have similar isotopic compositions (but see “Timing,” below). In contrast, for higher organisms with differentiated cells, it is entirely possible that two compounds with identical carbon skeletons and biosynthetic origins (same pathway of synthesis, same location within the cell) but from different cell types (leaf epidermis \textit{vs} palisade cell) would have very different isotopic compositions even though both were products of the same organism.

\textbf{Timing and reversibility}

The concept of steady state was stressed in the discussion of Figures 2-5. But even bacterial populations have phases of growth (lag, exponential, stationary, and death) and, within a single cell, the production of one or more enzymes need not be uniform over the life of a cell. As a result, isotope effects at one or more important branch points may change. If an enzyme with a small isotope effect substitutes for one with a large effect, the isotopic compositions of downstream products will change. A specific example of this has been identified in methanotrophic bacteria (Summons \emph{et al}., 1994) and is discussed in a concluding section of this review.

Reversibility of carbon flows must also play a role in controlling isotopic compositions of biosynthetic products. If carbon flows only to a particular product, it will be necessary only to consider isotope effects and branching ratios on the synthetic pathway. On the other hand, the isotopic compositions of compounds that are constantly being degraded as well as produced – compounds which “turn over” within the organism – will be shaped in addition by the isotopic characteristics of the degradative processes. Moreover, the degree of reversibility will be important. Molecular catalysts for which needs change over the lifetime of an organism – enzymes – turn over rapidly. Structural components such as lignin, cellulose, and some proteins (built from the same amino acids also found in enzymes) turn over much more slowly. There is evidence that the flow of carbon to the fatty acids in bacterial membranes is essentially unidirectional (Cronan and Vagelos, 1972) and that some fatty acids involved in the regulation of membrane fluidity in higher plants turn over quite rapidly (Monson and Hayes, 1982b), perhaps in response to diurnally cycling temperatures.

\textbf{Isotopic Compositions of Compound Classes Relative to Biomass}

The most basic sorting of isotopes occurs in the distri-
cbit of carbon among nucleic acids, proteins, carbohydrates, and lipids, the major classes of compounds present within most cells. The most important pathways of carbon flow are indicated schematically in Figure 6. In autotrophs, these lead from carbohydrates (the direct products of carbon fixation) to proteins, nucleic acids, and lipids. Biomass – the organic material that comprises a living organism – is a mixture of these products. Two kinds of isotopic relationships are often discussed, those between various classes (e. g., “lipids are depleted in $^{13}$C relative to proteins”) and those between classes and biomass (e. g., “abundances of $^{13}$C in nucleic acids and in biomass are essentially equal”). The first of these comparisons expresses a relationship like that between $\delta_3$ and $\delta_4$ in Figure 4. As carbon flows either to lipids or to proteins, we can expect the isotopic difference between them to remain roughly constant even though the $\delta$ values themselves will change. That change occurs relative to the available carbon supply ($\delta_4$ in Fig. 4). Such changes must affect class-

- **Pentose sugars** in nucleic acids and related materials such as nucleoside cofactors, proteins and amino acids, mono- and polysaccharides, and lipids of all kinds. The isotopic compositions of compound classes reported by Blair et al. (1985), Coffin et al. (1990), Benner et al. (1987), and Sternberg et al. (1986) converge to yield estimates of $X_{CProt}/X_{CNAA}$ = 8.6 and discussed further regularities: at maximal rates of growth, $X_{CProt}$ approaches 0.54; if the rate of growth is limited by availability of nutrients, $X_{CProt}$ declines to values as low as 0.15 (with parallel declines in $X_{CNAA}$); at low light levels, $X_{CSacc}$ increases relative to $X_{CLip}$.

Regularities prevail also in isotopic relationships. Isotopic compositions of compound classes reported by Blair et al. (1985), Coffin et al. (1990), Benner et al. (1987), and Sternberg et al. (1986) converge to yield estimates of $\delta_{NA} = \delta_{Prot} \cdot \delta_{Prot} \cdot \delta_{Sacc} = -1\%e$, and $\delta_{Lip} = -6\%e$. Figure 7, which expresses the depletion of $^{13}$C in lipids relative to biomass, has been prepared using these relationships and equation 5. Note: the indicated depletions have been
calculated, not measured. The intent is to illustrate and to consider factors controlling $\delta_{\text{lip}} - \delta_{\text{biomass}}$. If the saccharide-to-lipid fractionation in a particular organism were appreciably different from $-6\%$, the vertical axis would expand or contract. The results show both that the depletion of lipids in $^{13}$C relative to biomass can vary widely and that, in nature, a consistent depletion can be understood quite readily. The dotted and solid lines represent relationships between the isotopic depletion and $X_{\text{CProt}}$ at varying values of the lipid/carbohydrate ratio ($X_{\text{CLip}}/X_{\text{CSacc}}$). They show that the isotopic depletion becomes more marked as lipids become less abundant (i.e., as $X_{\text{CProt}}$ increases) and that the relative abundance of carbohydrates can also be an important controlling factor.

The biomass of phytoplankton successfully competing in marine environments generally falls within the area enclosed by the gray envelope (Laws, 1991). Compositions can vary more widely in laboratory cultures and in some extreme environments. The range which has been observed is roughly indicated by the extent of the dotted lines and by the points and ranges indicated for Arctic and Antarctic phytoplankton (Laws, 1991 and references therein). For the particular case of phytoplankton in subtropical gyres, Laws (1991) estimates values of $X_{\text{CProt}}$ and $X_{\text{CLip}}/X_{\text{CSacc}}$ in the range indicated by the open circles. The maximum-protein end of this range lies close to the composition derived by Anderson (1995) as an updated and improved estimate of the Redfield composition ($C_{106}H_{175}O_{52}N_{16}P$ instead of $C_{106}H_{263}O_{110}N_{16}P$).

**Isotopic Compositions of Carbohydrates**

**Mechanisms of production**

Organic carbon is depleted in $^{13}$C relative to inorganic carbon largely because of isotopic fractionations associated with the fixation of CO$_2$. Moreover, some of the hydrogen incorporated in this process becomes the first non-exchangeable H that shapes the hydrogen isotopic composition of organic materials.

Not all carbon fixation is photosynthetic. Table 2 provides an overview that has been organized in terms of the substrates, enzymatic catalysts, and first stable products. It does not include all processes in which inorganic carbon is incorporated in organic molecules, but it is meant to include all processes that contribute to net carbon fixation in organisms that can grow autotrophically. It thus includes pathways that occur not only in plants but also in Bacteria and Archaea that build biomass from inorganic carbon while deriving energy from chemical reactions.

For the moment, we will focus on photoautotrophs that utilize rubisco. Rubisco is the official name of an enzyme for which the systematic name – ribulose-1,5-bisphosphate carboxylase oxygenase – is inconveniently long. As indicated by the first activity specified in the systematic name, this enzyme catalyzes the carboxylation of ribulose-1,5-bisphosphate, “RuBP,” a five-carbon molecule. A six-carbon product is formed as a transient intermediate, but the first stable products are two molecules of “PGA,” 3-phosphoglyceric acid, $C_3H_7O_7P$. The carbon number of this compound gives the process its shorthand name, “C$_3$ photosynthesis.” At physiological pH, the acidic functional groups on the reactants and products are ionized as shown below.

$$\text{CO}_2 + \text{PGA} \rightarrow 2\text{PGA}$$

This reaction fixes carbon but there is no net change in oxidation number. The CO$_2$ is reduced to carboxyl but one of the carbon atoms in the RuBP is oxidized to yield the second carboxyl group. In subsequent steps, each mole of PGA reacts with a mole of NADPH in order to produce two moles of 3-phosphoglyceraldehyde, a product in which average oxidation number of carbon is 0. NADPH is the reduced form of nicotinamide adenine dinucleotide phosphate (see any biochemistry text for structures and further details). In biosynthetic processes, it functions as a hy-
A typical reaction is shown below. Note that NADPH + H⁺ is equivalent to NADP⁺ + H₂.

\[
\text{NADPH} + \text{H}^+ + \text{H}_3\text{C}-\text{CH}_2\text{O} \rightarrow \text{H}_3\text{C}-\text{CH}_2\text{O} + \text{NADP}^+ (7)
\]

The isotopic composition of the H⁻ transferred from NADPH to biosynthetic substrates must be one of the most important factors controlling the hydrogen-isotopic composition of organic matter.

For contrast and completeness, NAD⁺ and NADH should be introduced here. NAD⁺ is the oxidized form of nicotinamide adenine dinucleotide. It is structurally identical to NADP⁺ except that it lacks a phosphate group at a key point. Order and control are brought to biochemical oxidations and reductions by this seemingly trivial distinction. NAD⁺ is generally an oxidant. NADPH is generally a reductant. Each is present within a cell at only microscopic

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### Table 2. Enzymatic isotope effects and overall fractionations associated with fixation of inorganic carbon during autotrophic growth

<table>
<thead>
<tr>
<th>Pathway, enzyme</th>
<th>Reactant and substrate</th>
<th>Product</th>
<th>εi, ‰</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rubisco, form I, green plants and algae</td>
<td>CO₂ + Ribulose-1,5-bisphosphate</td>
<td>3-Phosphoglyceric acid (two moles)</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>Rubisco, form II, bacteria and cyanobacteria</td>
<td>CO₂ + Ribulose-1,5-bisphosphate</td>
<td>3-phosphoglyceric acid (two moles)</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>Phosphoenolpyruvate (PEP) carboxylase</td>
<td>HCO₃⁻ + PEP</td>
<td>Oxaloacetate</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Phosphoenolpyruvate (PEP) carboxykinase</td>
<td>CO₂ + PEP</td>
<td>Oxaloacetate</td>
<td>30</td>
<td>1</td>
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<tr>
<td><strong>C4 and CAM</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Phosphoenolpyruvate (PEP) carboxylase</td>
<td>HCO₃⁻ + PEP</td>
<td>Oxaloacetate</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Rubisco, form I, green plants and algae</td>
<td>CO₂ + Ribulose-1,5-bisphosphate</td>
<td>3-Phosphoglyceric acid (two moles)</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td><strong>Acetyl-CoA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate dehydrogenase</td>
<td>CO₂ + 2 [H]</td>
<td>HCO₂⁻</td>
<td>52</td>
<td>8</td>
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<tr>
<td>Carbon monoxide dehydrogenase</td>
<td>CO₂ + [H] + H₂Pt-CH₃ + CoASH</td>
<td>CH₃COSCoA + H₂O + H₂Folate</td>
<td>52</td>
<td>8</td>
</tr>
<tr>
<td>Pyruvate synthase</td>
<td>CO₂ + Acetyl-CoA + 2 [H]</td>
<td>Pyruvate + CoASH</td>
<td>52</td>
<td>8</td>
</tr>
<tr>
<td><strong>PEP carboxylase</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td><strong>PEP carboxylase</strong></td>
<td></td>
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<tr>
<td><strong>Reductive or reverse C4 Cycle</strong></td>
<td>CO₂ + Succinyl-CoA</td>
<td>α-Ketoglutarate + CoASH</td>
<td>4-13</td>
<td>11</td>
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<tr>
<td>α-Ketoglutarate synthase</td>
<td>CO₂ + α-Ketoglutarate + 2 [H]</td>
<td>Isocitrate</td>
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<tr>
<td>Isocitrate dehydrogenase</td>
<td>CO₂ + α-Ketoglutarate + 2 [H]</td>
<td>Pyruvate + CoASH</td>
<td></td>
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<tr>
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<tr>
<td><strong>PEP carboxylase</strong></td>
<td></td>
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<td><strong>PEP carboxylase</strong></td>
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<td><strong>3-Hydroxypropionate Cycle</strong></td>
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<td>Acetyl-CoA carboxylase</td>
<td>HCO₂⁻ + Acetyl-CoA</td>
<td>Malonyl-CoA</td>
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<tr>
<td>Propionyl-CoA carboxylase</td>
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<td>Methylmalonyl-CoA</td>
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<td></td>
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<tr>
<td>unknown</td>
<td>HCO₂⁻ + Propionyl-CoA</td>
<td>Malonyl-CoA</td>
<td></td>
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</tr>
<tr>
<td>unknown</td>
<td></td>
<td></td>
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</tbody>
</table>

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For the C₃ and C₄ pathways, the enzymes are listed in the order in which they process carbon. For the bacterial pathways, the enzymes are listed in order of their quantitative contributions to net carbon fixation.

Notes:
1. The tabulated range is an estimate chosen to include both tropical phytoplankton with δ ≈ -18‰ and land plants with δ ≈ -30‰. 2. Guy et al., 1993; also reported as 29‰; Roeske and O’Leary, 1984. 3. Guy et al., 1993; also reported as 18‰; Roeske and O’Leary, 1985. 4. O’Leary et al., 1981. PEP carboxylase maintains supplies of C₃ metabolic intermediates (= anaplerotic fixation) during growth of C₃ plants and many bacteria. In C₄ plants it fulfills the same function and catalyzes the initial C-fixing reaction. 5. According to Descalas-Gros and Fontugne (1990), PEP carboxykinase is the main anaplerotic producer of C₄ intermediates in diatoms and chrysophytes. 6. The tabulated range is an estimate including both C₃ plants with δ = -10‰ and CAM plants with δ = -23‰. 7. Preuß et al. (1989); Belyaev et al. (1983). 8. Gelwicks et al. (1989), isotope effect observed at both methyl and carboxyl carbons of acetate. 9. Pyruvate synthase is distinct from pyruvate dehydrogenase, for which the isotope effect at C-3 is ca. 9‰ (Melzer and Schmidt, 1987). Both enzymes use thiamine pyrophosphatase as a cofactor. 10. Preuß et al. (1989) identify PEP carboxykinase as important in completion of the acetyl-CoA pathway in *Acetobacterium woodii*. 11. Preuß et al. (1989). 12. van der Meer et al. (2001b). 13. Although the immediate C-fixing processes yield malonyl- and methylmalonyl-CoA, the net product of the 3-hydroxypropionate cycle is glyoxylate, a C₂ molecule. The processes by which glyoxylate is assimilated and converted to biomass are not known (Strauss and Fuchs, 1993).
concentrations. Specialized mechanisms exist for the oxidation of NADH and for the reduction of NADPH. If they are not activated and do not continuously regenerate NADH or NADPH, the amount of oxidation or reduction that can occur is very strictly limited. The nicotinamide adenine dinucleotides are not the only biochemical reductants and oxidants, but they are by far the most common.

Rubisco’s second activity — oxygenase — can be troublesome. If concentrations of O₂ are high, as they are likely to be in a brightly illuminated plant, the reaction between RuBP and O₂ can compete with carboxylation (Zelitch, 1975). This process of photorespiration has a doubly negative result: CO₂ is not fixed and a mole of RuBP is destroyed. The problem is particularly severe for plants that grow in environments in which they must minimize amounts of water lost by evapotranspiration. If they close their stomata, they also tend to retain O₂ and to impede access to atmospheric CO₂. The ratio of CO₂ to O₂ declines over time. The problem is particularly severe for plants where CO₂ concentrations of CO₂ within them rise to levels as high as 1 mM, nearly 100x higher than the concentrations in equilibrium with atmospheric concentrations of CO₂. Rubisco now catalyzes the production of phosphoglyceric acid and the NADPH erived from the oxidation of the malate is used to reduce the PGA to 3-phosphoglyceraldehyde.

CAM plants use the same chemistry but package it differently. Specifically, they lack the “Kranz anatomy” that is the defining characteristic of the C₄ plants. Kranz is the German word for wreath and refers to the appearance — in a cross-sectioned leaf — of the cells which sheath the vascular bundles in C₄ plants. CAM stands for Crassulacean Acid Metabolism. There is no such thing as crassulacean acid. The name instead refers to the initial discovery of this pathway of carbon fixation, in which oxaloacetic, malic, and pyruvic acids play key roles, in plants from the family Crassulaceae. CAM plants open their stomata, take in CO₂, and produce malate at night. Temperatures and, consequently, water losses are lower. During the day, the stomata are closed and the malate is processed as in the bundle-sheath cells of C₄ plants. Diffusive losses of CO₂ are, however, greater than those in C₄ plants.

Very recently, it’s been demonstrated that some unicellular algae utilize the C₄ pathway of carbon fixation (Reinfelder, 2000). Accordingly, it’s necessary to distinguish clearly between C₄ plants, which have the Kranz anatomy, and the C₄ pathway, which is apparently quite widely distributed.

**The Calvin Cycle**

In all organisms that use rubisco to fix CO₂, whether initially (C₃ plants and most aerobic, chemoautotrophic bacteria) or after release of CO₂ from oxaloacetate (plants using the C₄ pathway), the 3-phosphoglyceraldehyde resulting from the reduction of the PGA is processed by the enzymes of the Calvin Cycle (synonyms = Calvin-Benson Cycle, Calvin-Bassham Cycle, reductive pentose phosphate cycle or pathway; Melvin Calvin received the Nobel Prize in chemistry in 1961 for his work on the pathway of carbon in photosynthesis – Andrew Benson and James Bassham were prominent among his coworkers). The reactions within the Calvin cycle are schematically summarized in Figure 8. As shown, RuBP is regenerated, an obvious requirement if carbon fixation is to be sustained. The reactions are complicated (Calvin and Bassham, 1962) but the overall plan is simple. The net result of numerous rearrangements (none of which involve oxidation or reduction of carbon) can be summarized by the equation:

\[ 3 \text{C}_3 + 3 \text{CO}_2 \rightarrow 6 \text{C}_3 \rightarrow 3 \text{C}_4 + \text{C}_3 \]  

Where C₃ represents RuBP and C₃ is a three-carbon carbohydrate, either glyceraldehyde or dihydroxy acetone. The C₃ can either be exported from the chloroplast directly, used for the synthesis of lipids and proteins within the chloroplast, or used to produce starch (a polymer of glucose, a C₆ carbohydrate) that can be retained in the chloroplast (in cells without chloroplasts, starch can occur in the cytosol).

**Isotopic fractionations, C₃ pathway**

The reaction network is shown below:
NADPH + H⁺ are required to provide the 12 electrons required to reduce carbon. PGA represents 3-phosphoglyceric acid. Subscripts Cs represent carbohydrates for which the net oxidation number is zero. Plants, various substitutions allow partial pressures of CO₂ (Farquhar, 1989). For aquatic emergent plants in which the rate-determining transport of CO₂ occurs in air and 0.8‰ for plants that grow under water (0.7‰, O’Leary, 1984; 0.87‰, Jähne, 1987). Rubisco exists in multiple forms with differing isotope effects (Robinson and Cavanaugh, 1995). Values of εrubisco are 29‰ for higher plants (Roeske and O’Leary, 1984), and apparently somewhat lower for most phytoplankton and bacteria (Roeske and O’Leary, 1985; Guy et al., 1993; Robinson and Cavanaugh, 1995; Popp et al., 1998b).

Isotopic fractions, C₄ pathway

The reaction network for a C₄ plant is shown below:

\[
\begin{align*}
\delta_A &= \varphi_1, \delta_1, \\
\delta_1 &= \varphi_3, \delta_3, \\
\delta_2 &= \varphi_2, \delta_2, \\
\delta_3 &= \varepsilon_l, \delta_1, \\
\delta_4 &= \varphi_5, \delta_5, \\
\delta_5 &= \varepsilon_l, \delta_4.
\end{align*}
\]  
\[
\begin{align*}
\delta_A &= \varepsilon_l, \\
\delta_1 &= \varepsilon_l, \\
\delta_2 &= \varepsilon_l, \\
\delta_3 &= \varepsilon_l, \\
\delta_4 &= \varepsilon_l, \\
\delta_5 &= \varepsilon_l.
\end{align*}
\]

Terms are defined in parallel with those in reaction 10, with the addition of \( \delta_C \), \( \delta_{C_4} \), and \( \delta_{C_5} \) which indicate respectively...
bicarbonate in equilibrium with CO₂ in mesophyll cells. C added to phosphoenolpyruvate (PEP) to produce malate and oxaloacetate, and CO₂ released by decarboxylation of oxaloacetate. Leakage of CO₂ from the site of decarboxylation is represented by φ₁. Taking this into account is a key factor if we are to understand variations in fractionation associated with the C₄ pathway. Two branch points appear in this network. For carbon in mesophyll cells we require

\[ \phi_1 \delta_1 + \phi_2 \delta_2 = \phi_1 \delta_1 + \phi_2 \delta_2 \]  

and, for carbon released by decarboxylation of oxaloacetate:

\[ \phi_1 \delta_1 = \phi_2 \delta_1 + \phi_2 \delta_2 \]  

Defining a leakage parameter, \( L = \phi_1/\phi_2 \), we obtain

\[ \delta_1 = L(\delta_1 - \delta_2) + \delta_2 \]  

Relating the isotopic compositions of the specified fluxes of carbon to those of the various carbon pools, we can write \( \delta_1 = \delta_1 - \epsilon_{P_4} \), \( \delta_2 = \delta_1 - \epsilon_p \), \( \delta_2 = \delta_1 - \epsilon_c \), and \( \delta_3 = \delta_1 - \epsilon_{tw} \), where \( \epsilon_{P_4} \) and \( \epsilon_{tw} \) are respectively the isotope effects associated with mass transport of CO₂ in air and in water (as specified above, 4.4 and 0.8‰, respectively). \( \epsilon_{P} \) is the equilibrium isotope effect relating bicarbonate and dissolved CO₂ (Mook et al., 1974), and \( \epsilon_c \) is the isotope effect associated with fixation of bicarbonate by phosphoenolpyruvate carboxylase (O’Leary et al., 1981; here we use a compromise value of 2.2‰ implied by O’Leary, 1981).

Using the relationships specified, our task is to rearrange and combine equations 14-16 in order to produce an expression for \( \epsilon_{P_4} (= \delta_2 - \delta_1) \), the net isotopic fractionation associated with the C₄ pathway. Using \( \delta_2 = \delta_2 \) = \( \epsilon_{P_4} \epsilon_{P} - \epsilon_{tw} \), equation 16 can be rewritten as \( \delta_1 = L(\epsilon_{P_4} - \epsilon_{tw}) + \delta_2 \). Substituting for \( \delta_2 \) yields an expression incorporating \( \epsilon_P \):

\[ \delta_1 + \epsilon_{P_4} - \epsilon_{tw} = L(\epsilon_{P_4} - \epsilon_{tw}) + \delta_2 \]  

Terms relating to the mass balance at C₁ must be incorporated and \( \delta_1 \), which can’t be measured, must be eliminated. To accomplish these steps, we substitute \( \delta_1 = \delta_1 + \epsilon_{ia} \), replacing \( \delta_1 \) by an expression obtained by rearranging equation 14. The result is

\[ \phi_1 \delta_1 + \phi_2 \epsilon_{ia} + \phi_2 \epsilon_{Tw} + \phi_2 (\epsilon_{ia} + \epsilon_{P_4} - \epsilon_{tw}) = \]  

\[ \phi_2 L(\epsilon_{P_4} - \epsilon_{tw}) + \phi_2 \delta_2 \]  

Division by \( \phi_2 \) yields important terms with the coefficient \( \phi_1/\phi_2 \), which is equal to \( 1 - f_2 \). A final substitution for \( \delta_1 \) based on rearrangement of equation 15 yields

\[ \delta_1 - \delta_2 = \epsilon_{P_4} = \epsilon_{ia} + [\epsilon_{i} + \epsilon_{P_4} + L(\epsilon_{P_4} - \epsilon_{tw}) - \epsilon_{ia}] (1 - f_2) \]  

This equation relates \( \epsilon_{P_4} \) to the minimum number of controlling variables, specifically the isotope effects and the branching ratios \( (L, f_2) \) within the reaction network. Earlier, Farquhar (1983) presented a physiologically based derivation of an expression for \( \epsilon_{P_4} \). Equation 19 is precisely equivalent except for the coefficient for \( L \). The earlier derivation adopted \( \epsilon_{tw} = 0 \) and thus yielded \( LE \) rather than \( L(\epsilon_{P_4} - \epsilon_{tw}) \). Expressed in terms of the internal and ambient partial pressures of CO₂, \( 1 - f_2 = p/p_a \).

Two important aspects of the carbon isotopic fractionation imposed by the C₄ pathway are summarized graphically in Figures 9 and 10. The first schematically indicates the carbon-isotopic relationships between dissolved CO₂, bicarbonate, and the carbon that is added to phosphoenolpyruvate in the reaction catalyzed by phosphoenolpyruvate carboxylase. It shows that, because the kinetic isotope effect associated with PEP carboxylase is smaller than the equilibrium isotope effect between dissolved CO₂ and bicarbonate, the fixed carbon is enriched in 13C relative to that in the dissolved CO₂. As result, the CO₂ subsequently made available to rubisco in CAM and C₄ plants is enriched in 13C relative to atmospheric CO₂. If that carbon were fixed with perfect efficiency, the biomass of the plant would be enriched in 13C relative to CO₂ from the atmosphere – \( \epsilon_{P_4} \) would be negative, indicating an inverse fractionation.

Values of \( \delta_1 \) and \( \delta_2 \) (see reaction 13) are, however, influenced strongly by the branching ratios at the C₃ and C₄ branch points. The first pertains to exchange of CO₂ between the atmosphere and the plant. As long as the
partial pressure of CO₂ in the interior air spaces of the leaf is not zero, CO₂ will be diffusing out of the plant as well as into it. The second pertains to leakage of CO₂ away from the site at which oxaloacetate is decarboxylated. Depending on whether that leakage is minimal or extreme, the isotope effect associated with rubisco will be expressed to a lesser or greater degree at the C₅ branch point. Net fractionations are plotted as a function of L and p/pₐ in Figure 10. For all cases, p/pₐ = 0 corresponds to a situation in which carbon fixation is limited entirely by the transport of CO₂ into the plant. Accordingly, p/pₐ = 0 requires εₕ₄ = εₖ₄ = 4.4‰, independent of L. If no CO₂ leaked away from the site of decarboxylation of oxaloacetate but, at the same time, the plant was very freely exchanging CO₂ with the atmosphere – an implausible combination of circumstances corresponding to p/pₐ = 1 and L = 0 – maximal inverse fractionation would result, with the fixed carbon being enriched in ¹³C relative to atmospheric CO₂ by 5.7‰ (for εₚₐₙₐ = 7.9‰ at 25°C). In contrast to these extremes, maize, a representative C₄ plant, typically has L = 0.27 and p/pₐ = 0.4 (Marino and McElroy, 1991). Consistent with these values, measurements of the fractionation between atmospheric CO₂ and cellulose from corn kernels consistently yielded εₕ₄ = 3.28‰ (Marino and McElroy, 1991). Given the weak dependence of εₕ₄ on p/pₐ (a hypothetical C₄ plant with L = 0.38 would yield εₕ₄ independent of p/pₐ), Marino and McElroy (1991) proposed that C₄-plant debris could provide a record of the isotopic composition of atmospheric CO₂. For CAM plants, which lack the specialized, bundle-sheath cells found in C₄ plants, values of L are significantly higher and, as a result, isotopic depletions are significantly greater.

Figure 11. Relationships between the hydrogen isotopic compositions of initial photosynthate and related products and of water available within plant cells. The uncertainty indicated on the pathway between water and photosynthate reflects the possibility of offsetting fractionations in the production of NADPH and the subsequent transfer of H⁺ to photosynthate.

Hydrogen is introduced by the reduction of PGA, with NADPH serving as the hydride donor (cf. reaction 7). As shown in Figure 8, the fixed carbon and its accompanying, “non-exchangeable” hydrogen (the H directly bonded to C) are then shuffled among C₃, C₄, C₅, C₆, and C₇ sugars. The net effects are regeneration of ribulose biphosphate and repackaging of fixed C and H into trioses (glyceraldehyde, dihydroxyacetone) and hexoses (fructose, glucose). These products can be used in biosynthesis, exported to the cytosol, or stored within the chloroplast as starch. The inventory of C-bound H remains constant within the Calvin Cycle. Isotopic fractionations can, therefore, only affect the distribution of D among positions, not the overall δ value. The hydrogen can, however, be partly exchanged with H₂O in the course of the numerous rearrangements within the Calvin Cycle. Yakir and DeNiro (1990) have reviewed earlier work and have experimentally isolated the production of photosynthate well enough to estimate εₚₚₘ = -171‰, where P designates photosynthate and w the water used by the plant. That isotopic relationship between water and photosynthate is indicated schematically at the left of Figure 11. The sketch includes some broken lines and a question mark to indicate that the fractionation might represent the net of two or more processes. These could include the transfer of H from H₂O to NADP⁺, possibly favoring H that was strongly depleted in D (Luo et al., 1991); the transfer of H from NADPH to PGA; and subsequent exchanges of C-bound H with H₂O, a process which is known to favor partitioning of D into the carbohydrate (1.00 ≤ K ≤ 1.18; Cleland et al., 1977).

There are ample opportunities for further hydrogen-isotopic fractionations. These have been well studied for the specific case of the non-exchangeable H in cellulose. As demonstrated by the examples cited in Table 3, they attenuate the fractionation imposed during the initial production of photosynthate. None of the values of εₚₚₘ is as large as 171‰. Yakir (1992) has reviewed evidence show-
Figure 12. The flow of H from initial photosynthate (C₃ within the chloroplast) to stored carbohydrates and to cytosolic NADPH. Asterisks mark processes in which exchange of C-bound H with water is likely. B, biomass; NA, nucleic acids; (aa), minor contribution to amino acids.

Figure 13. Flow of carbon from dihydroxyacetone phosphate to phosphoglycerate (and, with oxidation, to 3-phosphoglyceric acid and biomass) and to C sugars. As shown, fructose-1,6-biphosphate can be formed by combining dihydroxyacetone phosphate and phosphoglycerate. The further sugar shown has the structure of glucose (stereochemistry not shown). Carbon positions in such aldohexoses are numbered 1-6, starting with the aldehydic carbon. The processes indicated are possibly related to the intramolecular distribution of ¹³C in glucose observed by Rossmann et al. (1991).

The evidence for isotopic inhomogeneity is substantial. The carbon-isotopic fractionations described thus far have pertained to fixed carbon entering the Calvin Cycle. The extensive transfers of carbon within that cycle might be expected to act as an isotopic mixer, so that the distribution of ¹³C within carbohydrates from photosynthate would be uniform. To examine this hypothesis, Rossmann et al. (1991) used chemical degradations and microbial fermentations to produce CO₂ from each of the six carbon positions within glucose. One sample of glucose was from corn starch (maize, a C₄ plant) and the other was from sucrose from sugar beets (C₃). Both samples represented cytoplasmic, storage forms of carbohydrate and both yielded closely similar results. Relative to the average for the molecule, positions C-3 and C-4 were enriched in ¹³C by 2 and 5‰, respectively, and position C-6 was depleted by 5‰. The precision of the analyses was such that the apparent imbalance (more enrichment than depletion) is not significant, but the consistency between plants and between the chemical and fermentative degradations is impressive. The evidence for isotopic inhomogeneity is substantial.

The relevant reaction network is shown in Figure 13. The input to the system is dihydroxyacetone phosphate, exported from the chloroplast. In the cytosol, its equilibration with glyceric acid-3-phosphate is catalyzed by triosephosphate isomerase. As the first step in respiratory metabolism or in biosyntheses requiring C₂ units, a portion of the glycerol-3-phosphate is dehydrogenated to yield PGA. Rossmann et al. (1991) suggest that an isotope effect associated with this reaction could lead to enrichment of ¹³C at the aldehyde carbon in glycerol-3-phosphate and that, by action of the triose phosphate isomerase, the enrichment could propagate to the free-alcohol carbon in dihydroxyacetone phosphate. Since these carbons flow to positions C-4 and C-3, respectively, in glucose, the mechanism provides a good explanation for the isotopic enrichment at those positions, even accounting for the greater enrichment at C-4. The depletion of ¹³C at C-6 is unexplained. Rossmann et al. (1991) speculate that it arises because C-6 is sheltered from all of the bond-making and -breaking in the oxidative pentose phosphate pathway, which provides an independent means of affecting isotopic compositions within glucose. This might be the beginning of a correct explanation, but C-5 is also relatively sheltered and seems not to be isotopically unusual.

### Intramolecular carbon-isotopic order in carbohydrates

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### Isotopic Compositions of Amino Acids

Amino acids are unique in that the first report of their isotopic compositions also provided intramolecular analyses (n. b., intramolecular = position-specific). In fact, the paper by Abelson and Hoering (1961) introduced the concept of intramolecular isotopic order and thus established a new basis for understanding the isotopic compositions of
organic compounds. The breakthrough resulted in part from the development of ion-exchange-chromatographic techniques for the separation of amino acids. This high-resolution liquid chromatographic technique provided quantities that were large enough for isotopic analyses using the techniques available in 1960. In conventional analyses, amino acids were detected by mixing the column effluent stream with a solution of ninhydrin. The resulting reaction, essentially quantitative (Moore and Stein, 1951), produces a strongly colored product that can be detected spectrophotometrically. In their preparative separations, Abelson and Hoering spiked initial hydrolysates with traces of radiocarbon-labeled amino acids and followed them through the separations. Peaks were collected and aliquots were treated with ninhydrin:

\[
\begin{align*}
\text{Asx} + \text{CO}_2 + \text{R-C}^3\text{O} + 3\text{H}_2\text{O} & \rightarrow \\
& \text{Asx} + \text{CO}_2 + \text{R-C}^3\text{O} + 3\text{H}_2\text{O}
\end{align*}
\]  

As shown in the equation above, CO₂ is produced from the carboxyl group of the amino acid. Because the reaction is essentially quantitative, the isotopic composition of the CO₂ specifically reflects the abundance of ^{13}C in the carboxyl group.
The tricarboxylic acid cycle (TCA cycle) provides a logical starting point. Relationships between carbon skeletons in that cycle are shown in Figure 16. The TCA cycle is normally thought of as the central carbon-processing facility of respiratory metabolism. As shown, acetyl groups are transferred from coenzyme A to oxaloacetate in order to produce citrate. The citrate is oxidatively decarboxylated to yield α-ketoglutarate which is then oxidatively decarboxylated to yield succinate. The succinate is further oxidized to yield oxaloacetate, thus regenerating the reactant needed for the next turn of the cycle. In effect, acetate is burned to yield CO₂. The oxygen required comes from H₂O. The redox budget is balanced by removal of H₂ (in the form of NADH + H⁺, not shown in Figure 16). A separate system (the "electron-transport chain") uses an inorganic electron acceptor (O₂, NO₃⁻, SO₄²⁻, among others) to oxidize the H₂ and conserves the energy produced by the oxidation of the acetate. Quite apart from its role in oxidative metabolism, the TCA cycle is important to amino-acid biosynthesis because, by producing oxaloacetate and α-ketoglutarate, it provides the carbon skeletons for aspartic and glutamic acids (notably the amino acids most strongly enriched in ¹³C, see Fig. 14).

The key step in amino-acid biosynthesis is production of the carbon skeleton rather than the amino acid itself. Given the carbon skeleton, the process is completed by transamination. An example is shown in reaction 21.

\[
\text{H}_2\text{C}_2\text{O}_4 + \text{Glutamate} \rightarrow \text{Aspartate} + \text{NH}_4^+ \quad (21)
\]

In this case, the availability of phenylpyruvic acid allows production of phenylalanine. As shown, amino groups are generally provided by glutamic acid. A special system exists for the production of glutamate from α-ketoglutarate + NH₄⁺. With this basic information we can now consider specific biosyntheses.

The aspartic-acid family can be considered first. The relationship of aspartic acid to oxaloacetate (OAA) and thus to the TCA cycle creates a problem, particularly for growing photoautotrophs. Aspartic acid is a very common amino acid. Relatively large quantities are required for the synthesis of proteins. If all the OAA is used to make aspartate, the TCA cycle will be shut down because citrate is required. As shown in Figure 16, this is provided by the carboxylation of phosphoenolpyruvate (PEP), which can be formed directly from the products of C₃ photosynthesis. The resulting isotopic relationships are indicated schematically in Figure 17, which considers a representative C₃ plant with δ₁₃C = -8 and δ₁₅N = -25‰. The internal CO₂ will have δ₁₃C = 2‰ (from δ₁₅N = δ₁₃C + δ₁₅N, see eq. 11 and accompanying discussion) and the bicarbonate available to PEP carboxylase will be further enriched as a result of the isotope effect associated with the CO₂-equilibrium. The car-

Figure 16. A schematic view of the flow of carbon within the tricarboxylic acid cycle. The labels m, c, and b designate respectively carbon from the methyl and carboxyl positions of acetyl-CoA and from bicarbonate used to produce oxaloacetate from phosphoenolpyruvate. Inputs and outputs of water, of redox cofactors (NAD⁺, etc.), and of coenzymes (CoASH) have been omitted in order to focus on the carbon skeletons. The amination of α-ketoglutarate will be further enriched as a result of the isotope effect associated with the CO₂-equilibrium.
Figure 17. Diagram summarizing isotopic relationships between inorganic carbon pools and carbon in photosynthate and C4 carbon skeletons. $\varepsilon_{as}$ is the equilibrium isotopic fractionation between dissolved CO$_2$ and bicarbonate (Mook et al., 1974). $\varepsilon_{as}$ is the isotope effect associated with carbon fixation. The estimate of $\delta$ for internal CO$_2$ is based on $\varepsilon_{photosynthate} = -25\%$ and $\varepsilon_{as} = 27\%$. $\varepsilon_{as}$ is the isotope effect associated with phosphoenolpyruvate carboxylase (O’Leary et al., 1982). The carbon pools represented in the right-hand column are, from top to bottom, the carbon added by the carboxylation of phosphoenolpyruvate, the total carbon in aspartic acid and in oxaloacetate, and the total carbon in pyruvate.

The pathways leading to other members of the aspartic acid family are shown in Figure 18. Isotopic compositions of three of these, thr, ile, and lys, are shown in Figure 14. Threone (thr) retains all of the C present in asx. Why is it not similarly enriched? In fact, thr, ile, and lys are all significantly depleted in $^{13}C$ relative to asx. The first branch point occurs at thr itself, which can be used either to produce other amino acids or to synthesize proteins. A large isotope effect at C-4 in the production of aspartyl-b-phosphate could wholly or partly neutralize enrichment of $^{13}C$ at that position. But an isotope effect associated with the attachment of C-1 to aspartyl-transfer RNA via an ester linkage, the first step in protein synthesis, could lead to enrichment of $^{13}C$ at C-1. The second branch point occurs at $\beta$-aspartyl semialdehyde, which can flow either to homoserine or, with addition of pyruvate, to a seven-carbon intermediate that is rapidly cyclized to form 2,3-dihydropicolinate. Fractionation of this point could explain the depletion of $^{13}C$ in thr relative to asp, but would have the effect of sending a $^{13}C$-enriched stream toward lys, which is in fact even more strongly depleted. The 2,3-dihydropicolinate is later opened to yield diaminopimelate. The reactions leading from $\beta$-aspartyl semialdehyde to diaminopimelate add carbon that dilutes the enrichment at C-4 in asp and involve very significant changes in bonding at four carbon atoms. These processes and a further branch point at which diaminopimelate can be used to produce peptidoglycan (of which it is an important constituent in Gram-negative bacteria and in cyanobacteria) must play a role in explaining the depletion of $^{13}C$ in lys. A third branch point occurs at homoserine. Both paths involve largely irreversible reactions at the OH group (phosphorylation, acylation).
Significant isotopic fractionations are not likely.

Isoleucine (ile) is the last member of the aspartic acid family represented in Figure 14. Two processes are potentially responsible for its depletion in $^{13}$C relative to thr. The first lies at the thr branch point (Fig. 18), where an isotope effect on the transamination leading to $\alpha$-ketobutyrate could lead to depletion of $^{13}$C at C-2 in that product. The second is the addition of an acetyl group to produce $\alpha$-aceto-a-hydroxybutyrate. The carbonyl position in the acetyl group is likely to be depleted in $^{13}$C (Melzer and Schmidt, 1987).

Two members of the $\alpha$-ketoglutarate family of amino acids are represented in Figure 14. Carbon in glu + gln (glx) is only slightly less strongly enriched in $^{13}$C than asx. The intramolecular analyses which, in the case of glx, refer only to C-1, indicate enrichment of nearly 20‰ at that position. Tracking this carbon backward through the TCA cycle leads to C-4 of OAA. Comparison to the carboxyl-group enrichment measured for the sum of positions C-1 and C-4 in asp (Fig. 15) suggests that most of the excess $^{13}$C in OAA must have been at C-4. As indicated in Figure 19, at least four pathways lead away from newly synthesized glu. Any kinetic isotope effect associated with transamination (in which glu serves as the NH$_2$ source for the synthesis of other amino acids) would enrich C-2. Any isotope effect associated with production of glutamate-5-phosphate has the potential to enrich C-5. While both of these mechanisms might contribute to the enrichment observed in glu, the first step leading toward ornithine and ultimately to arginine involves a reaction at the amino group and is not likely to cause any carbon-isotopic fractionation. Moreover, from that point, no carbon is removed from the set of atoms flowing to arg and only one, for which serves as the source, is added. The depletion of $^{13}$C in arg is thus unexplained.

Valine (val), alanine (ala), and leucine (leu) are members of the pyruvate family. Their biosynthetic pathways are summarized in Figure 20. Pyruvate is closely related to the C$_3$ products of photosynthesis. At least one major pathway leading from it, the production of acetyl groups by pyruvate dehydrogenase, has a significant carbon kinetic isotope effect (Melzer and Schmidt, 1987). The slight enrichment observed in ala relative to the estimated isotope composition of photosynthetic fits into this picture nicely. As shown (Fig. 20), val is produced by addition of an acetyl group to the ala carbon skeleton. In spite of the expectation that this acetyl group would be depleted in $^{13}$C relative to the pyruvate, val is slightly enriched in $^{13}$C relative to ala. The branch point at $\alpha$-ketoisovalerate provides an opportunity for the required fractionation. A carbon kinetic isotope effect at C-2 in that intermediate would send a $^{13}$C-depleted stream to leu and a relatively enriched stream to val. The average content of $^{13}$C in leu would be further decreased by the addition of a $^{13}$C-depleted acetyl group. Leucine is the only amino acid which derives its carboxyl group from the carboxyl position in acetate. The uniquely large depletion of $^{13}$C in the carboxyl group of leu from heterotrophic organisms (Fig. 15) provided the first evidence for the large carbon kinetic isotope effect associated with pyruvate dehydrogenase, now held responsible for the depletion of $^{13}$C in n-alkyl lipids (DeNiro and
Epstein, 1977; Monson and Hayes, 1982a). Notably, the carboxyl group of leu from photoautotrophs is not commonly depleted in $^{13}$C (Fig. 14). It follows that the overall depletion of $^{13}$C in leu must be due also to depletions at other sites within the molecule.

Serine (ser) and glycine (gly) are members of the phosphoglycerate family, for which biosynthetic pathways are summarized in Figure 21. A close isotopic relationship to photosynthate would be expected, but ser is enriched by roughly 5‰ (Fig. 14; even larger enrichments have been reported by Abelson and Hoering, 1961, and by Winters, 1971). Particularly because there is no evidence for strong enrichment of $^{13}$C in the carboxyl group (Fig. 15), attention is due to the reaction by which gly is produced from ser. In it, C-3 of serine is transferred to tetrahydrofolate, from which it flows to provide C1 units in a wide variety of biosynthetic reactions. A significant isotope effect associated with the transfer would be consistent with the enrichment of $^{13}$C in ser relative to gly and with the relationships of these products to photosynthate.

The aromatic amino acids, phe and tyr remain. Their carbon derives from phosphoenolpyruvate and erythrose-4-phosphate. As required by the large, structural differences between those materials and the aromatic amino acids, there are many reactions between the biosynthetic feedstocks and the ultimate products. All but one of these, however, affect both phe and tyr which, as shown in Figure 22, share an immediate precursor. It is not possible to explain the intermolecular difference of more than 10‰ based on fractionations localized at the OH-bearing carbon in prephenate. The products each contain nine carbon atoms so that the required isotopic difference at that position would be 90‰. Pools of prephenate separated in time or space are indicated.

**Amino acids not from cultured cyanobacteria**

Strikingly different isotopic distributions are found when amino acids from multicellular plants or from natural populations of algal unicells are analyzed. In general, the trend to greater downstream depletion discernible in Figure 14 is less marked. The generally flatter isotopic distribution is, however, interrupted by some notable enrichments and depletions. These points, along with some notable inconsistency and variability, are exemplified by the distributions shown in Figures 23 and 24. Amino acids in the sequence from asx to ala are commonly depleted and those in the sequence from ala to lys are commonly enriched relative to the trend defined by *Anabaena*. One feature of this flattening is removal of the puzzling isotopic contrast between phe and tyr. Notable enrichments of $^{13}$C appear frequently in gly. On the other hand, the depletion of $^{13}$C in leu noted originally in the *Anabaena* data appears to be a robust feature.

The turnover of amino acids in microbial cultures increases in stationary phase. If it is, at least, more important during slow growth and under natural stresses, it fol-
The nucleic acids are polynucleotides. Each nucleotide contains one phosphate group, a C₅ sugar (ribose or deoxyribose), and an aromatic, heterocyclic "base," either a one-ring pyrimidine (four or five carbon atoms + N, O, and H) or a two-ring purine (five carbon atoms + N, O, and H). The carbon isotopic compositions of nucleic acids, therefore, are expected to represent an average of the ribose and the bases. In the four-carbon pyrimidines uracil and cytidine, three carbons come from C-2, 3, and 4 from asp and the fourth comes from carbamoyl phosphate (see structure in Fig. 19). The five-carbon pyrimidine thymidine adds one C from a tetrahydrofolate carrier (cf. discussion of the biosynthesis of glycine). The inclusion of C-4 from asp and the insertion of C from carbamoyl phosphate, for which asp serves as the carbon source, both suggest that the pyrimidines, with the possible exception of thymidine, would be enriched in ¹³C relative to photosynthate. The purines contain both carbons from gly, two C₁ units from THF, and a C from CO₂. If one dared to make a prediction about such a salad, it would be that it was depleted in ¹³C relative to photosynthate.

Observations indicate that the enrichments in the pyrimidines (likely, but of unknown magnitude) and the depletions in the purines (hypothetical, but apparently needed to explain the results) balance at least roughly. Pioneering analyses of the carbon-isotopic compositions of bacterial nucleic acids were reported by Blair et al. (1985), who found nucleic acids enriched in ¹³C relative to biomass by 0.6‰. More recently, this work has been very nicely extended by Richard Coffin and his coworkers, who report that bacterial nucleic acids are enriched relative to biomass by about 0.3‰ (Coffin et al., 1990). The isotopic compositions closely follow those of heterotrophic carbon sources, and this can be exploited to provide new information about the roles of bacteria in aquatic food webs (Coffin et al., 1994; 1997).
products are often preserved in sediments as porphyrins. As shown in Figure 25, its immediate precursor is aminolevulinic acid (C₅) which is in turn synthesized from glycine and succinyl-CoA with loss of CO₂ (C₂ + C₄ → C₅ + C₁) or from glutamate (C₅). The carbon flowing to tetrapyrroles is thus closely related to that in amino acids and in intermediates from the TCA cycle and is expected to have an isotopic composition close to that of biomass. In specific analyses of chlorophyllides (i.e., the non-phytol carbon from chlorophyll) from the photosynthetic bacteria *Rhodopseudomonas capsulata* and *Chromatium vinosum*, Takigiku (1987) found enrichments of 0.0 and 0.7‰ relative to biomass. Chloroplasts from beech tree leaves used to test procedures in the same investigation also yielded chlorophyllides enriched in 13C relative to plastid biomass by 0.7‰. Madigan *et al.* (1989) found chlorophyllides from *Chromatium tepidum* enriched in 13C by 0.5‰ relative to biomass. Considering diverse analyses that provided indirect evidence about the isotopic compositions of chlorophyllides relative to biomass, Laws *et al.* (1995) and Bidigare *et al.* (1999) concluded that 0.5‰ was a good estimate of the enrichment of chlorophyllides relative to biomass in marine plankton. In a single investigation of chlorophyllide from a cyanobacterium, *Synechocystis* sp., Saka**ta *et al.* (1997) found an enrichment of 2.7‰ relative to biomass and attributed the difference to shifted carbon flows that increased the abundance of proteins relative to lipids and other cellular components in cyanobacteria.

**Isotopic Compositions of Lipids**

Lipids have either linear or isoprenoidal carbon skeletons. All of the linear skeletons derive from the same biosynthetic pathway but there are two ways to produce isoprenoids, one of which has been discovered so recently that it is not mentioned in standard textbooks. The linear, or n-alkyl, lipids are often termed “acetogenic” because of their relationship to acetate, which is both their immediate biosynthetic precursor and the main product of their metabolic degradation. Structural variations within the acetogenic lipids are exemplified in Figure 26. Products containing no hydrolyzable linkages are “simple lipids,” those containing one or more ether or ester bonds are “complex lipids.” As shown, structural variations that affect the carbon skeleton are restricted to methyl branching and rare, cycloalkyl substituents.

The structural range of isoprenoidal carbon skeletons is far greater. All are based on the isoprene, but there are two means of variation: (1) the connections between isoprene units, whether head-to-tail, head-to-head, tail-to-tail, or irregular (involving isoprene positions C-2 or C-3); and (2) cyclization. Only the first of these is considered in Figure 27. Assuming that no carbon atoms have been added to or trimmed from the basic carbon skeleton, the carbon number for any isoprenoid is some multiple of five. A C₁₀ isoprenoid is a monoterpene, a C₁₅ is a sesquiterpene, a C₂₀...
is a diterpene... etc. In eukaryotic plants, including phytoplankton, the C_{20} and C_{40} isoprenoids are formed in the chloroplast and the C_{30} products are of cytosolic origin. The head-to-head linkage is generally restricted to the Archaea.

Turning to cyclization, isolated rings are common in C_{30} isoprenoid structures (carotenoids and archaeal tetraethers) but are not key points of classification. Structures with more than two fused rings are almost exclusively restricted to isoprenoids based on squalene (gibberellins, based on phytol, are a rare exception). Two major classes – tetracyclic and pentacyclic – are shown in Figure 28. Sterols are tetracyclic structures that are important constituents of all eukaryotic membranes. Although they have been widely reported as present in heterotrophic bacteria and cyanobacteria (see, for example, references cited by Kohl et al., 1983), proven instances of their biosynthesis are limited to aerobic, methanotrophic bacteria (Bird et al., 1971; Summons et al., 1994) and to *Nannocystis exedens* (Kohl et al., 1983). The emphasis on biosynthesis, demonstrated by transmission of an isotopic label from a precursor to steroidal products stems from (1) the demonstration by Levin and Bloch (1964) that sterols isolated from cyanobacterial cultures were not biosynthetic products but instead contaminants apparently derived from the media and (2) the observation that some bacteria which incorporate sterols in their membranes nevertheless require an exogenous source for the sterolic carbon skeleton (Razin, 1978). Pentacyclic isoprenoid alcohols, often with highly polar, non-lipid substituents that raise their carbon number to 35 or more, are produced by many aerobic bacteria and apparently serve as sterol surrogates in their membranes (Rohmer et al., 1984). The dominant family of products is based on the hopane carbon skeleton (Fig. 28). Two additional groups of pentacyclic triterpenoids are comprised entirely of six-membered rings. Those with an OH group on the fifth ring are commonly products of protozoans. Tetrahymanol (Fig. 28) is a prominent example (Raederstorff and Rohmer, 1988). A thus-far-unique bacterial occurrence of this compound has also been reported in *Rhodopseudomonas palustris* (Kleeman et al., 1990). Pentacyclic triterpenoids with an OH group on the first ring are commonly products of higher plants.

**Processes affecting the carbon-isotopic compositions of n-alkyl lipids**

*n*-Alkyl carbon skeletons are essentially acetate polymers derived from acetyl-coenzyme A. In plants and in heterotrophs, that building block is formed by the oxidative decarboxylation of pyruvate, which is produced by the degradation of carbohydrates (including the immediate, C_{3}}
products of photosynthesis). The reaction is catalyzed by pyruvate dehydrogenase and is associated with a significant, carbon kinetic isotope effect (Melzer and Schmidt, 1987). Because pyruvate has multiple fates, isotopic fractionation is expected and, in fact, the general depletion of $^{13}$C in $n$-alkyl lipids has been attributed to this step (DeNiro and Epstein, 1977; Monson and Hayes, 1982a). Additional factors must be important and may help to explain variations in lipid isotopic compositions (Blair et al., 1985). These factors include variations in the branching ratios at pyruvate; alternate sources of acetyl-CoA, which is also produced by the degradation of some amino acids (ile, leu, thr, and trp); and downstream isotope effects. Acetyl-CoA itself has multiple fates. If the reaction pathways competing for it have different isotope effects, the isotopic compositions of the $n$-alkyl lipids will vary from that of the acetyl-CoA. The fates include oxidation to CO$_2$ via the TCA cycle; biosynthesis of some amino acids (leu, lys); the production of $n$-alkyl lipid carbon skeletons; the production of mevalonic acid, a precursor of isoprene; and, in many aquatic unicells, hydrolysis to yield acetate which leaks from the cell before being utilized (Roberts et al., 1955; Blair et al., 1985).

The site of fatty-acid synthesis varies very significantly. If a cell has chloroplasts – if it is an algal unicell or a carbon-fixing cell within a differentiated photoautotroph – all fatty acids with 18 or fewer carbon atoms are produced within it (Cavalier-Smith, 2000). If a cell lacks chloroplasts the fatty acids are produced in the cytosol using (in eukaryotic cells) acetate exported from the mitochondria. The consequences of these points can be discussed more completely and systematically in parallel with a consideration of isoprenoid lipids.

**Processes affecting the carbon-isotopic compositions of isoprenoid lipids**

The isoprene carbon skeleton is indicated schematically in Figure 27. The corresponding biosynthetic reactant – equivalent in its role to acetyl-CoA – is isopentenyl pyrophosphate. As shown in Figure 29, this compound can be made by two different and fully independent pathways. The mevalonic-acid pathway was until recently thought to be the only route to isoprenoids. The deoxyxylulose-phosphate, or methyerythritol-phosphate, pathway was first discovered in Bacteria by Rohmer and coworkers (Flesch and Rohmer, 1988; Rohmer et al., 1993). Subsequent investigations (reviewed by Lichtenthaler, 1999) have shown that this pathway is widely distributed in prokaryotes, in the chloroplasts of eukaryotic algae and higher plants, and in the cytosol of members of the Chlorophyllae (including the Trebouxiophyceae, Chlorophyceae, and Ulvophyceae; see summary in Table 4 and note very recent clarification by Schwender et al., 2001). In contrast to acetyl-CoA, isopentenyl pyrophosphate is a dedicated product flowing only to the biosynthesis of a single class of lipids. The isotopic compositions of the polyisoprenoid lipids will therefore be controlled by the isotopic composition of the isopentenyl pyrophosphate.

![Figure 29. Relationships between the carbon positions in isopentenyl pyrophosphate and their sources.](image)
Isotope effects potentially associated with the syntheses of mevalonic acid or of methyl-erythritol have not been directly investigated. Nor have intramolecular patterns of isotopic order been measured in polyisoprenoids. As a result, stepwise or process-related isotopic fractionations associated with the biosynthesis of polyisoprenoid lipids can only be estimated from observed isotopic compositions of whole molecules.

**Carbon isotopic compositions of lipids from heterotrophic bacteria**

Four separate investigations of carbon isotopic fractionation in aerobic growth of *Escherichia coli* provide consistent and complementary results that provide a foundation for understanding the isotopic compositions of lipids. DeNiro and Epstein (1977) grew separate cultures of *E. coli* using glucose, pyruvate, and acetate as carbon sources. “Lipids” produced by the bacteria were depleted in $^{13}C$ by 6-8‰ relative to glucose or pyruvate but nearly unfractonated relative to acetate. Accordingly, they concluded that the reaction that produced acetate from pyruvate must be responsible for the depletion of $^{13}C$ in lipids. To examine that process specifically, they made site-specific analyses of all of the carbon positions in the reactants and products, substituting readily available yeast pyruvate carboxylase for *E. coli* pyruvate dehydrogenase. They found normal kinetic isotope effects at all carbon positions in pyruvate: $\varepsilon_{C-1} = 7.8$, $\varepsilon_{C-2} = 14.7$, and $\varepsilon_{C-3} = 1.0%$. C-2 in pyruvate becomes the carboxyl carbon in acetyl-CoA. C-3 becomes the methyl carbon. Because of the strongly differing isotope effects, depletion of $^{13}C$ at the carboxyl position should be 15 times greater than that at the methyl position. The chain of chemically indistinguishable CH$_2$ groups within the fatty acid should contain two isotopically distinct subsets of carbon atoms, with those derived from the carboxyl carbon being depleted in $^{13}C$ relative to those derived from the methyl carbon. The depletion of $^{13}C$ in the molecule overall would be the average of the small depletion at the even-numbered positions and the large depletion at the odd-numbered positions.

Monson and Hayes (1980, 1982a) grew *E. coli* on glucose, isolated the individual fatty acids, and used chemical techniques to obtain CO$_2$ from specific positions within the molecules. Key reactions produced CO$_2$ quantitatively from carboxyl groups (Vogler and Hayes, 1978; 1979) and oxidized double bonds quantitatively to produce carboxyl groups at the terminal positions of cleavage products (Monson and Hayes, 1982a). The double bonds in unsaturated fatty acids produced by *E. coli* do not result from the action of a desaturase enzyme. Instead, even when O$_2$ is available, they are produced by an anaerobic mechanism that prevents complete hydrogenation of the alkyl chain as it is lengthened during biosynthesis. No mechanism exists for the isotopic fractionation of the doubly bonded carbons – for example, at C-9 and C-10 in n-hexadec-9-enoic acid – relative to the other odd- and even-numbered carbon positions in the molecule. Like DeNiro and Epstein (1977), Monson and Hayes (1982a) found that the crude lipids were depleted in $^{13}C$ by 7%$^\circ$ relative to the carbon supplied by glucose. The individual fatty acids were, however, depleted by only 3%$^\circ$ (the difference being explained by the presence of more strongly depleted neutral components in the lipid fraction). Production of CO$_2$ from positions within the carbon chain showed that the odd-numbered carbon positions, derived from the carboxyl group of acetyl-CoA, were depleted relative to the supplied glucose by 6 ± 1‰ and that the even-numbered carbon positions, derived from the methyl position of acetyl-CoA, were enriched by 0.5 ± 1.4‰. These results decisively confirmed the existence of intramolecular isotopic order in n-alkyl lipids. The pattern, moreover, was consistent with the outline provided by De-
Niro and Epstein (1977) in that fractionation was localized at the carboxyl carbon of acetyl-CoA.

To interpret their results quantitatively in terms of isotope effects and reaction pathways, Monson and Hayes (1982a) adopted DeNiro and Epstein’s (1977) most basic finding and considered the branch point shown in Figure 30. The existence of well-supported estimates of the carbon flows (Roberts et al., 1955) allowed construction of a fractionation plot based on the assumption that the isotopic composition of C-2 in the pyruvate was equal to that of the glucose supplied to the culture. The resulting fractionation plot based on the assumption that the isotopic composition of acetyl-CoA was unaffected by any isotopic fractionation occurred on the pathway leading from acetyl-CoA to fatty acids and that the steady-state loss rate indicated in Figure 30 would be the net of those opposing flows. An isotope effect associated with the assimilation of acetate may, therefore, play a role.

In the final investigation, Melzer and Schmidt (1987) simply made a good measurement of the isotope effects associated with pyruvate dehydrogenase from E. coli. For $\varepsilon_{C-2}$ they reported 21‰, in excellent agreement with the estimate provided by Monson and Hayes (1982a). In sum, these four investigations provide a roughly quantitative view of the processes responsible for the depletion of $^{13}$C in n-alkyl lipids but also show how many factors might have importances that are not yet understood.

Isotopic fractionations in lipid biosynthesis during aerobic and anaerobic growth of *Shewanella putrefaciens* on lactate have recently been compared by Teece et al. (1999). Under aerobic conditions, fatty acids were depleted in $^{13}$C relative to biomass by 2-3‰, roughly duplicating the characteristics of E. coli discussed above. In contrast, fatty acids produced under anaerobic conditions were depleted in $^{13}$C by 5-10‰ relative to biomass. Earlier, Scott...
and Nealson (1994) had concluded that, under anaerobic conditions, \textit{S. putrefaciens} metabolized lactate to yield acetyl-CoA and formate, with most of the acetate being excreted and a portion of the formate being assimilated \textit{via} the serine pathway. If the latter pathway were fully functional, it would provide a second source of acetyl-CoA. The carboxyl carbon in that product is derived from \textit{via} PEP carboxylase, but Teece et al. (1999) do not report the isotopic composition of the CO$_2$ in their cultures. Together, the metabolic and isotopic investigations provide an excellent demonstration of anaerobic-bacterial complexity and its related hazards. Good work has led to good data but not to firm conclusions. The wide range of depletions (5-10‰) might indicate varying contributions from the two sources of acetyl-CoA (\textit{i.e.}, lactate and the serine pathway). The relatively large depletion must be kept in mind when considering the isotopic compositions of fatty acids in natural systems.

**Carbon isotopic compositions of lipids from cyanobacteria**

Although prokaryotic and thus uncompartmentalized, cyanobacteria differ from the organisms just considered in that they are photosynthetic, and thus obtain from light much of the energy required to drive biosynthesis. Moreover, they produce isoprenoidal as well as \textit{n}-alkyl lipids. Sakata et al. (1997) recently reported results of the first investigation of lipid-biosynthetic fractionations in a cultured cyanobacterium, specifically \textit{Synechocystis}, which uses the MEP pathway for synthesis of isoprenoids (Lichtenthaler et al., 1997; Disch et al., 1998).

Extractable \textit{n}-alkyl lipids were depleted in $^{13}$C relative to total biomass by 9.1‰, a fractionation three times greater than that in \textit{E. coli}. If localized at the odd-carbon positions, the depletion of 8.4‰ would require $f_{AcCoA} = 0.22$ (see Fig. 30). Factors suggested as responsible for the 3.4-fold decrease in $f_{AcCoA}$ were (i) much lower needs for acetyl-CoA in energy production \textit{via} the TCA cycle and (ii) very low concentrations of lipids in \textit{Synechocystis} (2% of biomass C). These are good points, but it will not be surprising if further investigations show that the large depletion is due to multiple factors.

Three subsets of polisoprenoids, shown in Figure 31, were analyzed. Phytol, which accounted for 90% of the total polisoprenoids and 1% of biomass C, was depleted relative to biomass by 6.8‰. Diploptene and diplopterol, comprising 0.04% of biomass C, were similarly depleted (6.9, 6.5‰). Bishomohopanol (0.2% of biomass C), a degradation product of bacteriohopanepolyol, was depleted by 8.5‰. The latter result is striking because, as noted in Figure 31, bishomohopanol contains two carbons that are derived from carbohydrate (Rohmer, 1993) and which are expected to be enriched in $^{13}$C relative to lipid carbon. It suggests that the triterpenoid portion of the bishomohopanol is significantly more strongly depleted (≈ 9‰) than the other isoprenoids. Noting that phytol, diploptene, and diplopterol were all resident mainly in membranes and that the abundance of bacteriohopanepolyol was more strongly correlated with cellular volume (Jürgens et al., 1992), Sakata et al. (1997) attributed the difference to changing branching ratios over the life of the cell, with greater depletion being favored in the later (volume-correlated) products.

The results provide some information about the isotopic characteristics of the MEP pathway of isoprenoid biosynthesis. As shown in Figure 29, there are two processes by which MEP might be depleted in $^{13}$C relative to photosynthetic. The first is in the decarboxylation of pyruvate to yield the C$_2$ unit which is condensed with glycerol to yield deoxyxylulose. The second – if, as seems likely for a C$_4$ carbohydrate, the deoxyxylulose has alternate fates – is in the rearrangement of the linear carbon skeleton to yield methylerythritol. If only the first of these steps were effective, only the tertiary C would be depleted in each isoprene unit and, to account for the observed overall depletion of 6.8‰, the depletion at that position would be 34‰. Since this exceeds the isotope effect found or estimated for any enzyme-catalyzed decarboxylation of pyruvate, it appears very likely that further fractionation, affecting at least one if not two additional carbon positions, is associated with the second step.

**A survey of carbon isotopic compositions of lipids from other organisms**

The lipid fractionations discussed in the preceding paragraphs provide the first entries in Table 5, which also summarizes results from many further investigations and provides a basis for discussion.

Lipids from several photosynthetic bacteria have been studied (photosynthetic bacteria are anaerobic phototrophs that use electron donors other than water and which are distinct from cyanobacteria). The Chromatiaceae (\textit{Chromatium, Thiocapsa}) can grow phototrophically (using light energy but an organic carbon source) as well as...
autotrophically. As autotrophs they use rubisco and the Calvin Cycle to fix carbon and to produce photosynthate (T. roseopersicina is reported to use in addition the reversed TCA cycle; Ivanovsky, 1985). The corresponding fractionations of isoprenoids summarized in Table 5 must refer to biosyntheses via the MEP pathway, which is assumed to be universal in Bacteria. There is no obvious reason that C. tepidum, C. vinosum, Rhodopseudomonas capsulata, and T. roseopersicina should fractionate less strongly than Synechocystis (≈ 4 vs. 6-8‰) and the similar fractionation obtained when C. tepidum uses acetate as the carbon source is remarkable, given the relatively complex network of reactions produced to produce biosynthetic intermediates under such conditions (typically the glyoxalate cycle, Madigan et al., 2000, p. 629). Van der Meer et al. (1998) note that the inverse fractionation (= enrichment) of 13C in n-alkyl carbon skeletons produced by T. roseopersicina may be due to use of acetate produced by the reversed TCA cycle (see discussion of C. limicola, below).

Chemoautotrophs are microbes which obtain energy by catalyzing an exergonic chemical reaction (commonly at interfaces between aerobic and anaerobic environments) and which produce biomass by fixing inorganic carbon. *Nitrosomonas europea* oxidizes ammonia and uses rubisco and the Calvin Cycle to fix carbon (the most common sulfide-oxidizing organisms also use the Calvin Cycle). Its isotopic characteristics could therefore, be expected to be similar to those of other aerobic, prokaryotic autotrophs such as *Synechocystis*. As shown in Table 5, this expectation is fulfilled.

Nearly all of the remaining organisms listed in Table 5 do not produce C3–C6 carbohydrates as immediate products of carbon fixation nor do they commonly assimilate them (M. capsulatus is the exception). As a result, pathways of carbon flow differ strongly from those in the organisms already discussed. *Chlorobium limicola*, for example, is a member of the Chlorobiaceae, which use the reversed TCA Cycle for fixation of carbon. Van der Meer et al. (1998) point out that the flow of carbon in such organisms proceeds from CO2 to acetate and then to C3 and larger compounds. Since each further step brings a chance for more fractionation, it should not be surprising that n-alkyl carbon skeletons are enriched in 13C relative to biomass, although the magnitude of the enrichment in comparison to that observed in isoprenoids is both remarkable and unexplained.

*Chloroflexus aurantiacus* fixes carbon using the 3-hydroxypropionate pathway (Strauss and Fuchs, 1993). Remarkably, it is known also to produce its isoprenoids from the MVA pathway (Rieder et al., 1998). Van der Meer et al. (2001b) point out that, if the same pool of acetate is used to produce the n-alkyl lipids, the isotopic compositions at the methyl and carboxyl positions of the acetate groups used in biosynthesis can be calculated (e.g., there are 12 methyl and 8 carboxyl carbons in the C20 isoprenoid and 8 of each in the C16 fatty acid). The fractionations reported in Table 5 then indicate that the carboxyl carbon is enriched in 13C relative to the methyl carbon by 40‰. The pathways of carbon flow in *C. aurantiacus* are very incompletely known and the branch points poten-

### Table 5. Observed depletions of 13C in lipids relative to biomass, prokaryotes

<table>
<thead>
<tr>
<th>Organism</th>
<th>C source</th>
<th>Metabolism</th>
<th>n-Alkyl</th>
<th>Isopren.</th>
<th>Reference</th>
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<tr>
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<td>glucose</td>
<td>het. O2</td>
<td>3</td>
<td></td>
<td>Monson and Hayes, 1982</td>
</tr>
<tr>
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<td>glucose</td>
<td>het. O2</td>
<td>3</td>
<td></td>
<td>Blair et al., 1985</td>
</tr>
<tr>
<td>S. putrefaciens</td>
<td>lactate</td>
<td>het. O2</td>
<td>2-3</td>
<td></td>
<td>Teece et al., 1999</td>
</tr>
<tr>
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<td>lactate</td>
<td>het. CO2</td>
<td>5-10</td>
<td></td>
<td>Teece et al., 1999</td>
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<tr>
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<td>9</td>
<td>6-8 E</td>
<td>Sakata et al., 1997</td>
</tr>
<tr>
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<td>C3</td>
<td>3.5 E</td>
<td></td>
<td>Madigan et al., 1989</td>
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<td>5 E</td>
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</tr>
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<td>C3</td>
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<td>C3</td>
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<td>rev. TCA</td>
<td>-16 -11</td>
<td>-2 -3 E</td>
<td>van der Meer et al., 1998</td>
</tr>
<tr>
<td>T. roseopersicina</td>
<td>CO2</td>
<td>C3 + rev. TCA</td>
<td>-4 -2</td>
<td>4-5 E</td>
<td>van der Meer et al., 1998</td>
</tr>
<tr>
<td>C. aurantius</td>
<td>CO2</td>
<td>3-OH prop.</td>
<td>0.7</td>
<td>4 V</td>
<td>van der Meer et al., 2001b</td>
</tr>
<tr>
<td>N. europea</td>
<td>CO2</td>
<td>C3 NH3/O2</td>
<td>7-13</td>
<td>9.3 E</td>
<td>Sakata et al., 2001</td>
</tr>
<tr>
<td>T. ruber</td>
<td>CO2</td>
<td>unknown</td>
<td>-2</td>
<td></td>
<td>Jahnke et al., 2001</td>
</tr>
<tr>
<td>M. capsulatus</td>
<td>CH6</td>
<td>RuMP</td>
<td>2-4</td>
<td>6-11 E</td>
<td>Summons et al., 1994</td>
</tr>
<tr>
<td>M. capsulatus</td>
<td>CH6</td>
<td>RuMP</td>
<td>2-4</td>
<td>6-10 E</td>
<td>Jahnke et al., 1999</td>
</tr>
<tr>
<td>M. trichosporium</td>
<td>CH6</td>
<td>serine</td>
<td>12</td>
<td>2 E</td>
<td>Jahnke et al., 1999</td>
</tr>
<tr>
<td>C. aurantius</td>
<td>CH6</td>
<td>RuMP</td>
<td>5</td>
<td>6.4 E</td>
<td>Jahnke et al., 1999</td>
</tr>
<tr>
<td>M. barkeri</td>
<td>CH6</td>
<td>(CH3)3</td>
<td>M′gen</td>
<td>18 V</td>
<td>Summons et al., 1998</td>
</tr>
<tr>
<td>M. barkeri</td>
<td>CH6</td>
<td>(CH3)3</td>
<td>M′gen</td>
<td>29 V</td>
<td>Summons et al., 1998</td>
</tr>
<tr>
<td>M. thermoautotrophicum</td>
<td>CH2</td>
<td>3-OH prop.</td>
<td>-2</td>
<td></td>
<td>Takigiku, 1987</td>
</tr>
<tr>
<td>M. sedula</td>
<td>CO2</td>
<td>unknown</td>
<td>-2</td>
<td></td>
<td>van der Meer et al., 2001a</td>
</tr>
</tbody>
</table>

* For genera see text.  † E = MEP, V = MVA.  ‡ 17:1 unique at -5‰.  ‡ 17:0 unique at 4‰.  † Tabulated depletions pertain only to end products formed early in life of a culture, when only the membrane-bound form of methane monooxygenase (MMO) is present. Squalene is enriched relative to end products. Fractions decrease when soluble MMO is also present.
fractionally responsible for such a large fractionation cannot be identified.

*Thermocrinus ruber* is a hyperthermophilic, hydrogen-oxidizing chemooautotroph. The pathway by which it assimilates inorganic carbon is unknown. Its biomass is depleted relative to CO$_2$ by only $3\%\epsilon$. The $n$-alkyl lipids are in turn slightly enriched relative to biomass so that their isotopic composition is very close to that of the CO$_2$. This case, fractionations may be minimized both by the high temperature, which reduces the magnitude of equilibrium isotope effects, and by the structure of the metabolic reaction network, which apparently leads to similar isotopic compositions for the precursors of both amino acids and lipids.

The remaining entries in Table 5 are involved in the production and consumption of methane. *Methylococcus capsulatus*, *Methylosinus trichosporium*, and the isolate designated as CEL1923 are methylotrophic bacteria. Such organisms assimilate methane (and, in many cases, other “C$_1$ compounds,” such as methanol, formaldehyde, formic acid, methyl amines, and methyl sulfides) using either a pathway that yields carbohydrates (the ribulose monophosphate or RuMP pathway) or one which yields acetyl-CoA after the C$_3$ unit is initially added to glycine to yield serine (thus called the serine pathway). The methyl carbon in that acetyl-CoA derives from the C$_3$ substrate but the carboxyl carbon derives from CO$_2$. Variations are possible within the serine pathway. 3-Phosphoglyceric acid, an oxidation product of serine, can be withdrawn from the pathway much as C$_2$ and C$_3$ acids are withdrawn from the TCA cycle (Jahnke et al., 1999). The acetyl-CoA product can reenter the cycle and lead to the production of succinate for use in biosyntheses (White, 1995, p. 265).

The RuMP- and serine-pathway methylotrophs yield sharply contrasting lipid-biosynthetic fractionations. In *M. capsulatus*, the $n$-alkyl carbon skeletons are depleted by 2-4$\%\epsilon$ and the isoprenoids are depleted by 6-11$\%\epsilon$. This resembles patterns seen in other prokaryotes in which biosynthesis starts from carbohydrates. The $n$-alkyl carbon skeletons are depleted as in *E. coli* and the isoprenoids are depleted roughly as in *Synechocystis* and *N. europaea*. But *M. capsulatus* does not have a complete TCA cycle. Processes unique to the RuMP-cycle methylotrophs must be controlling the isotopic compositions of the lipid precursors in *M. capsulatus*. The serine pathway used by *M. trichosporium* leads straightforwardly to acetyl-CoA but not to building blocks for isoprene units. Why, then, are the $n$-alkyl carbon skeletons from serine-cycle methanotrophs so much more strongly depleted than the isoprenoids? If phosphoglyceric acid is withdrawn from the pathway and condensed with a C$_2$ unit to obtain deoxyxylulose, where is that C$_2$ unit coming from? It cannot be the same acetyl-CoA that is yielding the strongly depleted $n$-alkyl carbon skeletons. Very interestingly, the strong depletion in the $n$-alkyl carbon skeletons resembles that in anaerobic cultures of *Shewanella*, in which serine-pathway metabolism has been invoked on the basis of enzymological studies (Scott and Nealson, 1994).

The initial study of *M. capsulatus* (Summons et al., 1994) provided the best example of temporal variations in isotopic fractionations. Two variants of methane monooxygenase, one soluble, the other membrane-bound, mediate the assimilation of methane. The membrane-bound enzyme is dominant at low cell densities (i. e., during the exponential phase of growth) and has an isotope effect that is large compared to that of the soluble enzyme, which becomes important later. As a result, all of the carbon initially available for production of biomass is depleted in $^{13}$C relative to that assimilated later. The mixture of lipids produced also changes as the culture ages. Specifically, 3-methyl bacteriohopanepolyol and 4,4-dimethylsterols increase in abundance relative to bacteriohopanepolyol and 4-methyl sterols. As a result of these changes, the late-synthesized methyl hopanoids and dimethyl sterols are enriched in $^{13}$C relative to the nonmethylated hopanoids and monomethyl sterols even though all derive from the same precursor (namely, squalene). The effect could be observed only by harvesting cultures at varying cell densities and making repeated analyses, and that very laborious task was undertaken only after initial observations (based, of course, on large amounts of material from fully grown cultures) had yielded “impossible” results (e. g., monomethyl sterols strongly depleted in $^{13}$C relative to their dimethyl homologs). These elegant investigations also showed the squalene itself was commonly enriched in $^{13}$C by 5-7$\%\epsilon$ relative to its products, indicating that an isotope effect significant even for a 30-carbon molecule must be associated with the cyclization reaction.

Individual lipids have been isotopically analyzed in only three Archaea, all of them methanogens (see entries for *Methanosarcina Barkeri*, *Methanococoides Burtonii*, and *Methanobacterium thermoautotrophicum* in Table 5). All reflect strong depletion of $^{13}$C in lipids relative to biomass. Methanogens are commonly described as fixing carbon by use of the acetyl-CoA pathway. This can provide the feedstock required for synthesis of lipids, but C$_3$ and C$_4$ carbon skeletons are required for the synthesis of amino acids (which, in the form of proteins, account for most of the biomass). In methanogens, these are produced by additional CO$_2$-fixing steps (White, 1995, p. 261). If the isotope effects associated with those reactions are much smaller than those associated with the production of acetyl-CoA, the isotopic contrast between the lipids and the biomass can be accounted for.

The entries in Table 6 pertain to eukaryotic algae. *S. communis, T. minimum, C. monotica*, and *Dunaliella* sp. are green algae and are expected to synthesize isoprenoids via the MEP pathway in the cytosol as well as in the plastids. The remaining species are expected to utilize the MEP pathway within the chloroplast and the MVA pathway in the cytosol. By far the most thorough and precise investigation was that of biosynthesis in *E. huxleyii* undertaken...
Depletions of $^{13}$C in the C$_{37}$ alkenones produced by *E. huxleyi* are of particular interest and illustrate a key point. The abundances of the alkenones relative to other products vary substantially depending on conditions of growth. For example, Riebesell et al. (2000) found that the alkenones comprised 1.0% of biomass C at low concentrations of dissolved CO$_2$ and up to 5.9% of biomass C at higher concentrations of dissolved CO$_2$. In spite of this variation in abundance, the depletion of $^{13}$C relative to biomass in the C$_{37}$ alkenidene consistently averaged 5.4‰ (s. d. = 0.3‰, n = 10) and was not correlated with [CO$_2$(aq)]. If, on the other hand, the isotopic composition of the same compound was expressed relative to that of the fully saturated C$_{16}$ fatty acid it varied systematically from a depletion of 2.5‰ at low concentrations of dissolved CO$_2$ to a depletion of 4.0‰ at high concentrations, apparently reflecting a progressive redistribution of carbon within the lipid-biosynthetic reaction network. Although they found a significantly different fractionation (4.2 vs. 5.4‰, light and nutrient limitations varied between the two treatments), Popp et al. (1998a) found that the isotopic composition of the C$_{37}$ alkenidene relative to biomass did not vary systematically with rate of growth although the isotopic composition of the biomass relative to the source CO$_2$ varied strongly.

Patterns of isotopic depletion among compound classes for other species frequently differ. It appears common, but far from universal, for MVA-pathway sterols to be depleted relative to biomass by 5-8‰. Phytol is nearly always less strongly depleted (2-5.5‰). Both of these relationships are consistent with field data summarized by Popp et al. (1999). If extended (≥ C$_{18}$)-n-alkyl carbon skeletons are abundant, they are often isotopically depleted relative to the C$_{14}$-C$_{17}$ fatty acids. Differences of more than about 1‰ cannot reasonably be attributed to the addition of a single, isotopically exotic acetyl unit (i.e., during the extension of a C$_{16}$ chain to C$_{18}$). Such contrasts, therefore, probably indicate the use of distinct pools of acetate. There is a problem, however, in asserting that the lighter n-alkyl carbon skeletons must derive from cytosolic acetate, since the MVA-derived isoprenoids must also come from that pool and they are generally lighter still. To explore these relationships more securely, some immediate objectives can be defined. We need to determine how much of the variability evident among the eukaryotic algae is experimental (analytical noise, stressed cultures under unnatural conditions, etc.) and how much is biological (response to subtle factors that may well vary in natural environments). We need to obtain a better view of the comparative isotopic characteristics of the MEP and MVA pathways. More analyses of sterols from green algae and any analyses of products of Euglena (in which the MVA pathway is found in both the plastid and the cytosol) would be helpful.

### Epilogue

It often seems that isotopic fractionations provide too much information about too many processes, combining it all in a package that is unmanageably intricate. In response, investigators keep increasing the complexity of the available data by providing more and more detailed analyses. The proliferation of compound-specific isotopic analyses is a prime example of this phenomenon. Does it increase the information-carrying capacity of the isotopic channel or is it another case of the triumph of entropy? To obtain the preferred result, we will have to understand biosynthetic fractionations like those reviewed here.

### Table 6. Observed depletions of $^{13}$C in lipids relative to biomass, eukaryotic algae

<table>
<thead>
<tr>
<th>Organism</th>
<th>n-Alkyl</th>
<th>Isoprenoids</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scenedesmus communis</td>
<td>7-9, 12</td>
<td>5.3 Ep, 7 Ec</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Tetraedron minimum</td>
<td>3-6, 6</td>
<td>0-5 Ep, 2 Ec</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Chlamydomonas monoica</td>
<td>7.5</td>
<td>4.9 Ep, 5.1 Ec</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Dunaliella sp.</td>
<td>3.5</td>
<td>0.5 Ep, 2.7 Ec</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Rhizosolenia setgera</td>
<td>5-7</td>
<td>2.9 Ep, 6-7 Vc</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Chaetoceros socialis</td>
<td>0-2</td>
<td>-1 Ep</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Thalassiosira weissflogii</td>
<td>1-2</td>
<td>1 Vc</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Gymnodinium simplex</td>
<td>1-3</td>
<td>4 Vc</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Isochrysis galbana</td>
<td>6-8, 3.1</td>
<td>2.8 Ep, 7 Vc</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Chrysochromulina polypleis</td>
<td>6</td>
<td>2.7 Ep</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Tetraselmis sp.</td>
<td>8</td>
<td>4.2 Ep, 0.4 Ec</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Rhodomonas sp.</td>
<td>5-7</td>
<td>2.2 Ep, 1 Vc</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>9, 7</td>
<td>3.4 Ep, 7 Vc</td>
<td>Bidigare et al., 1997</td>
</tr>
<tr>
<td>Emiliania huxleyi</td>
<td>6, 4.1</td>
<td>4.2 Ep, 7 Vc</td>
<td>Bidigare et al., 1997</td>
</tr>
<tr>
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<td>4.2</td>
<td>0.4 Ep, 5.4</td>
<td>Schouten et al., 1998</td>
</tr>
<tr>
<td>Emiliania huxleyi</td>
<td>2.5, 4.0, 5.4</td>
<td>2.0 Ep, 8.3 Vc</td>
<td>Riebesell et al., 2000</td>
</tr>
</tbody>
</table>

* In multiple entries, the first number or range pertains to C$_{14}$-C$_{18}$ Carbon skeletons presumably of plastidic origin and the second pertains to extended chains that may be of cytosolic origin.  
  
  1 Ep, 2 MVA, 3 plastid, 4 cytosol, 5 C$_{37}$ alkenidene only.  
  
  2 In sequence: C$_{14}$ and C$_{16}$ fatty acids, C$_{28}$ and C$_{30}$ fatty acids, C$_{32}$ fatty acid + C$_{37}$ and C$_{38}$ alkenidene.
Acknowledgements

Support for this work and for the author’s molecular-isotopic studies in general has come from the Programs in Exobiology and in Astrobiology at the National Aeronautics and Space Administration. It is a privilege also to acknowledge advice, comments, manuscripts, and reviews provided by Bob Bidigare, Marilyn Fogel, Ed Laws, Alex Sessions, Roger Summons, and Marcel van der Meer.

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