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Testing Models of Fatty Acid Transfer and Lipid Synthesis in Spinach Leaf Using in Vivo Oxygen-18 Labeling[1]

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Oxygen-18 labeling has been applied to the study of plant lipid biosynthesis for the first time. [$^{13}C_2{}^{18}O_2$]Acetate was incubated with spinach (*Spinacia oleracea*) leaves and the ^{18}O content in fatty acid methyl esters isolated from different lipid classes measured by gas chromatography-mass spectometry. Fatty acids isolated from lipids synthesized within the plastid, such as monogalactosyldiacylglycerol, show an ^{18}O content consistent with the exogenous acetate undergoing a single activation step and with the direct utilization of acyl-acyl carrier protein by the acyl transferases of the chloroplast. In contrast, fatty acids isolated from lipids assembled in the cytosol, such as phosphatidylcholine, show a 50% reduction in the ^{18}O content. This is indicative of export of the fatty acyl groups from the plastid via a free carboxylate anion, and is consistent with the acyl-acyl carrier protein thioesterase:acyl-coenzyme A (CoA) synthetase mediated export mechanism. If this were not the case and the acyl group was transferred directly from acyl-acyl carrier protein to an acyl acceptor on the cytosolic side, there would be either complete retention of ^{18}O or, less likely, complete loss of ^{18}O, but not a 50% loss of ^{18}O. Thus, existing models for fatty acid transfer from the plastid and for spatially separate synthesis of "prokaryotic" and "eukaryotic" lipids have both been confirmed.

The current model of export of fatty acids produced by de novo synthesis within the plastid states that acyl-acyl carrier protein (ACP) hydrolysis occurs in the plastid stroma, possibly at the inner leaflet of the inner envelope, and that the free fatty acid released is transferred to the outer envelope of the plastid where it is reactivated to acyl-CoA for utilization in non-plastidial acylglycerolipid synthesis. The chemical principle of this model was first proposed by Shine et al. (1976) as the basis of switching from ACP-thioester metabolism to acyl-CoA thioester metabolism in plant fatty acid synthesis. Subsequent experiments showed that ACP-dependent fatty acid synthesis was entirely a chloroplast function (Ohlrogge et al., 1979), implying that the acyl-ACP thioesterase was a stromal enzyme activity. Acyl-CoA synthetase was demonstrated as an activity of the chloroplast envelope (Roughan and Slack, 1977; Joyard and Stumpf, 1981), and later more specifically of the outer envelope (Andrews and Keegstra, 1983; Block et al., 1983). The assumption is that free fatty acids are the intermediates, although the mechanism by which such intermediates move is unknown. Certainly, the major product of fatty acid synthesis in isolated chloroplasts is free fatty acid, unless ATP and CoA are added, in which case acyl-CoA become a major end product (Roughan et al., 1979, 1980; Roughan and Slack, 1982).

The above model has not been tested by in vivo experimentation and there are other scenarios to describe fatty acyl export from the plastid. Thomas and co-workers have suggested that fatty acyl groups may be exported from the plastid as acyl carnitines (Thomas et al., 1982, 1983; McLaren et al., 1985). In addition, an acyl-CoA thioesterase was observed with chloroplast envelopes (Joyard and Stumpf, 1980) and was later more specifically localized to the inner envelope (Andrews and Keegstra, 1983; Block et al., 1983). The in vivo function of this thioesterase has remained unclear. The possibility of involvement in fatty acyl export can be considered if, during chloroplast isolation, a factor is lost that is required for this acyl-CoA thioesterase to actually function as a transacylase (with the loss of its cofactor its observed in vitro function is that of a thioesterase), or if it actually functions as an acyl-ACP transacylase, inner envelope acyl-transporter, and acyl-hydrolase in vivo.

From a large number of studies, including compositional analyses of chloroplastic and cytosolic lipids, leaf and chloroplast labeling experiments, and enzyme localization and substrate specificity studies, the prokaryotic/eukaryotic model for lipid assembly in the plant cell has been constructed (Roughan and Slack, 1982). This model has been more recently summarized to include information from the characterization of Arabidopsis mutants (Somerville and Browse, 1991). In this model, fatty acyl-ACPs can be used directly by plastid-localized acyl transferases for the synthesis of lysophosphatidic acid and then phosphatidic acid, which are intermediates in the synthesis of "prokaryotic" lipids that are assembled totally within the chloroplast (phosphatidylglycerol, and, in 16:3 plants, galactosyldiacylglycerol and sulfoquinovosyldiacylglycerol molecular species containing C16 fatty acids at the *sn*-2 position). Alternatively, the acyl-ACPs can provide acyl groups for export from the plastid for subsequent activation and assembly of phospholipids of the endomembrane system.

Incubations with [^{18}O]water have been used to assess deacylation-reacylation reactions mediated through hydrolytic intermediates for animal lipids (Wells, 1971; van Heusden and van den Bosch, 1979; Lombardo et al., 1986; Kuwae et al., 1987). When deacylation via hydrolysis occurs the resulting fatty acid will incorporate ^{18}O into the carboxylate anion. Subsequent reacylation will result in only a 50% loss of this ^{18}O incorporation. Apparently, ^{18}O

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stable isotope labeling has not been applied to analogous problems in plant lipid biochemistry. We therefore decided to test the model for fatty acyl group export from the chloroplast using a ^{18}O stable isotope method whereby the ^{18}O was initially incorporated into acetate, and hence to the carboxyl oxygen of long-chain acyl-ACPs. If the model for prokaryotic and eukaryotic lipid assembly is correct, no additional loss of ^{18}O would occur in the fatty acids isolated from prokaryotic lipids, whereas if fatty acids are exported from the plastid via a free carboxylate anion and reactivated prior to incorporation into eukaryotic lipids, these fatty acids would show a 50% loss of ^{18}O.

MATERIALS AND METHODS

Materials

[1,2-$^{13}C_2$]Acetic acid (99% ^{13}C) was purchased from Isotec (Miamisburg, OH); [1-^{13}C]acetic acid (99% ^{13}C) from Sigma (St. Louis) or Cambridge Isotope Labs (Andover, MA), and [^{18}O]water (70% ^{18}O) from Cambridge Isotope Labs. [1-^{14}C]Acetic acid (57.2 Ci/mol) was purchased from American Radiolabeled Chemicals (St. Louis).

Synthesis and Analysis of [$^{13}C_2{}^{18}O_2$]Acetic Acid

[1,2-$^{13}C_2$]Acetic acid (2 mmol) was combined with [^{18}O]water (17.5 mmol) and heated for 24 h at 75°C. The $^{16}O/^{18}O$ exchange reaction was monitored by gas chromatography-mass spectrometry (GC-MS) analysis of the mixture, diluted to 0.1% (v/v) in acetone. Acetic acid analysis was carried out using a model 5890 gas chromatograph configured with an autosampler and a model MSD 5972 mass analyzer (quadrupole, operating in electron impact mode, Hewlett-Packard, Palo Alto, CA). A DB-FFAP capillary column (15-m × 0.25-mm i.d., 0.25-μm thickness, J&W Scientific, Folsom, CA) was used, with helium carrier gas in "constant flow" mode, with splitless injection, and with both injector and MS interface temperatures set at 230°C. The initial GC oven temperature was 50°C for 3 min, followed by a rapid temperature ramp (20°C/min) to 180°C. The mass analyzer was run in scan mode from 10 to 100 atomic mass units. It was calculated from the reactant ratios that complete oxygen exchange would lead to acetic acid with 57.0 mol % ^{18}O compared with total ^{16}O plus ^{18}O. Figure 1 shows the mass spectrum of product obtained. The major peaks in the molecular ion cluster correspond to $^{13}C_2{}^{16}O_2$, $^{13}C_2{}^{16}O\ ^{18}O$, and $^{13}C_2{}^{18}O_2$ isotopic species, and give an ^{18}O content of 57.7%.

Incubations

Spinach (*Spinacia oleracea*) was grown hydroponically in the growth chamber at 25°C with an 8-h photoperiod. Incubations with acetate were performed with 2- to 4-mm-wide strips of rapidly expanding spinach leaves. Acetate, pH adjusted to 5.7 with NaOH, was incubated at 2 to 10 mM concentrations in 4.0 mL of 25 mM NaMES buffer, pH 5.7, with 0.3 to 0.4 g fresh weight of tissue, at 27°C and under illumination (300 μmol s^{-1} m^{-2}). Tween 20 was added as a wetting agent to a concentration of 0.0075% (w/v). At the end of the assay period, the tissue was quickly washed twice with de-ionized water to remove excess substrate and quenched by heating in isopropanol for 5 min at 80°C to 90°C. When necessary, aliquots of the incubation medium were removed during the assay and added to acetone (10 volumes) for direct GC-MS analysis of the acetic acid isotopic composition.

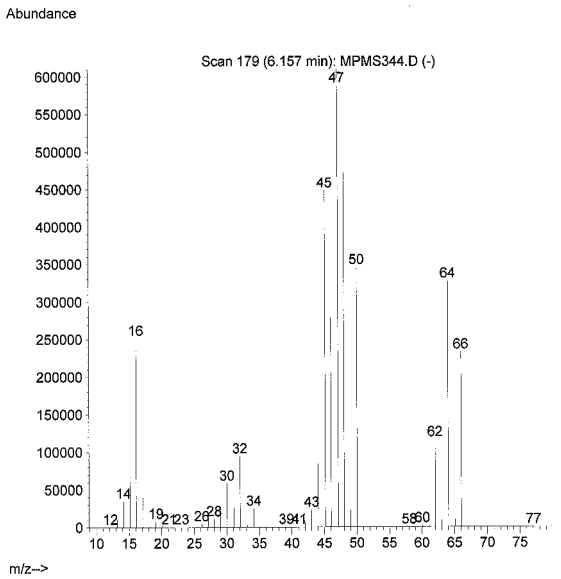

Figure 1. Mass spectrum of [$^{13}C_2{}^{18}O_2$]acetic acid, prepared at 99% ^{13}C and 58% ^{18}O enrichments. The major molecular ion peaks are at m/z = 62, corresponding to $^{13}C_2H_4{}^{16}O_2$; m/z = 64, corresponding to $^{13}C_2H_4{}^{16}O^{18}O$; and m/z = 66, corresponding to $^{13}C_2H_4{}^{18}O_2$. Minor molecular ion peaks are seen at m/z = 63, corresponding to $^{12}C\ ^{13}CH_4{}^{16}O^{18}O$; and m/z = 65, corresponding to $^{12}C\ ^{13}CH_4{}^{18}O_2$.

Lipid Analysis

Lipids were extracted from the quenched tissue with hexane-isopropanol, following the method of Hara and Radin (1978). An aliquot of the lipid extract was suspended in acetone:water (4:1, v/v) and A_{652} measured to determine chlorophyll (Arnon, 1949). Most incubations with acetate contained trace amounts of [1-^{14}C]acetic acid, even if their primary purpose was stable isotope labeling. An aliquot of the lipid extract was assayed by liquid scintillation counting to determine incorporation. Lipid classes and fatty acid methyl esters (FAME) were analyzed by thin-layer chromatography (TLC). Isolation of lipid classes and FAME by preparative TLC used the same systems. The TLC plate was scanned for radioactivity using an Instant Imager (Packard, Meriden, CT), both to quantitate radioactivity and to locate the appropriate bands for recovery from preparative TLC plates.

Polar lipids were analyzed on silica plates developed one-third and then two-thirds with chloroform:methanol:acetic acid:water (85:15:5:2, v/v/v/v); after thorough drying, they were developed fully with acetone:acetic acid:water (200:2:1, v/v/v). Polar lipids were eluted from the silica with chloroform:methanol:water (5:5:1, v/v/v) and

recovered after phase partitioning and evaporation of the chloroform layer. Molecular species of monogalactosyldiacylglycerol (MGDG) were separated by using C_{18}-reversed phase TLC and developing half and then fully with acetonitrile:methanol:water (130:70:1, v/v/v). FAME were prepared from the total lipid or isolated lipid classes by the base-catalyzed transmethylation method of Ichihara et al. (1996). Analysis and isolation of individual FAME were by either C_{18}-reversed phase TLC, developing half and then fully with acetonitrile:methanol:water (130:70:1, v/v/v), or by argentation-TLC using plates impregnated with a 5% (w/v) silver nitrate in acetonitile solution, dried, activated, and developed with hexane-diethyl ether mixtures. Intact MGDG and FAME were hydrogenated using hydrogen at slightly greater than atmospheric pressure with a platinum (IV) oxide catalyst in methanol. A two-hour reaction at room temperature gave complete reduction of unsaturated FAME to saturated FAME.

GC-MS Analysis of FAME and Determination of ^{18}O Content

FAME from assays with [1-^{13}C] or [$^{13}C_2$,$^{18}O_2$]acetic acid were analyzed by GC-MS using the system described above. Separations of FAME were carried out on a 30-m × 0.25-mm i.d., 0.25-μm thick DB-23 capillary column (J&W). The mass detector was operated in one of two modes, depending on the purpose of the experiment. In some cases the molecular ion cluster of the FAME was analyzed. In cases where the determination of ^{18}O content was required, this was undertaken by analysis of the ion cluster from the McLafferty rearrangement. Many aspects of the methodology are covered by Schmid et al. (1988).

For analysis of the molecular ion cluster, the detector was operated in single ion monitoring mode for ions $(M - 1)$ through $(M + n + 1)$, where M is the m/z value of the major natural isotopic abundance molecular ion and n represents the highest possible isotopic enrichment (50 ms dwell time per a.m.u., 1.3 scans/s). Since ionization is via electron impact, FAME molecular ion peaks are not highly abundant. Thus, for analysis of the molecular ion isotope cluster it was important for good signal-to-noise ratios to have GC samples with high concentrations of FAME (100–500 μg FAME/mL heptane). This necessitated concentration of methyl palmitate and methyl oleate by C_{18} reversed-phase TLC. Although these GC loadings gave overloaded peaks on the chromatograms, the mass spectra were of good quality.

For analysis of the McLafferty rearrangement ion cluster, the mass detector was operated in scan mode (60 to 80 atomic mass units, sampling no. 6, 3.3 scans/s) and sample concentrations of 20 to 200 μg FAME/mL heptane were used (typically 1–2 μL injected). Measuring the ^{18}O enrichments in saturated FAME is straightforward, since the McLafferty rearrangement peak at $m/z = 74$ is the base peak (100%) in the electron impact mass spectrum, where it typically represents about 30% of the total ion current, and since there are negligible interfering peaks in the region. However, for methyl oleate the McLafferty rearrangement peak is about 55% of the base peak, represents about 6% of the total ion current, and contains larger interfering peaks in the region that introduce inaccuracies into the measurement of isotope ratios when the combination $^{13}C/^{18}O$ labeling is used. The situation is worse still for methyl linoleate and methyl linolenate. To remove this problem, after isolation, all unsaturated FAME were hydrogenated prior to GC-MS analysis. As a control for hydrogenation, methyl [^{18}O]heptadecanoate, prepared synthetically, was taken through the hydrogenation protocol. The measured ^{18}O content was 39.95% prior to hydrogenation and 40.15% after hydrogenation, indicating that the hydrogenation protocol does not change the ^{18}O content of the FAME sample.

The McLafferty rearrangement ion at $m/z = 74$ [$^{12}C_3H_6{}^{16}O_2$, with a structure CH_2:C(OH)(OMe)] contains only C(1), C(2), and methoxy carbon atoms and the ester oxygens from the FAME. However, the methoxy oxygen atom is introduced by the base-catalyzed transmethylation, so only the carbonyl oxygen is of biosynthetic origin. After [$^{13}C_2{}^{18}O_2$]acetic acid labeling, the FAME containing an exogenous acetate unit at the carboxyl end will exhibit a McLafferty peak at 78 corresponding to $^{12}C^{13}C_2H_6{}^{16}O^{18}O$ unless the carbonyl oxygen has been exchanged during biosynthesis, in which case a peak at 76 corresponding to $^{12}C^{13}C_2H_6{}^{16}O_2$ will be observed. Example spectra of the McLafferty region are shown in Figure 2. The biosynthetic ^{18}O content of the FAME is defined as the intensity of $m/z = 78$/(intensity of $m/z = 76$ + intensity of $m/z = 78$), and is expressed as a percentage. The intensities of these peaks are first corrected by subtracting the natural abundance intensities for $m/z = 76$ and 78, relative to the base peak at $m/z = 74$ for unlabeled fatty acids. For unlabeled FAME the abundances of the $m/z = 76$ and 78 peaks relative to the $m/z = 74$ peak are effectively independent of the FAME concentration of the sample for GC-MS analysis.

Even in the saturated FAME mass spectrum there is a small cluster of ions, centered on $m/z = 69$ to 71 and representing C_5H_{9-11} ions, that if highly labeled with ^{13}C could cause overlapping peaks in the McLafferty region. Using a 50% contribution of exogenous [$^{13}C_2$,$^{18}O_2$]acetate to fatty acid synthesis, as discussed below for 10 mM acetate concentrations, and assuming a random distribution, it was calculated that the overlapping peaks would cause an actual 50.0% ^{18}O content to be measured as 49.0%. The correction factor for overlapping peaks would be in the same direction for all ^{18}O content measurements, is very small, and is therefore ignored in the measurement of ^{18}O content.

RESULTS

Experimental Design

To examine hydrolytic turnover of fatty acids in glycerolipids in vivo previous workers have used [^{18}O]water as a substrate and measured ^{18}O uptake into fatty acids (Kuwae et al., 1987). This method appeared problematic for leaf tissues, since ^{18}O from water will probably be introduced into the precursor supply for fatty acid synthesis by several mechanisms. These mechanisms include the bicarbonate

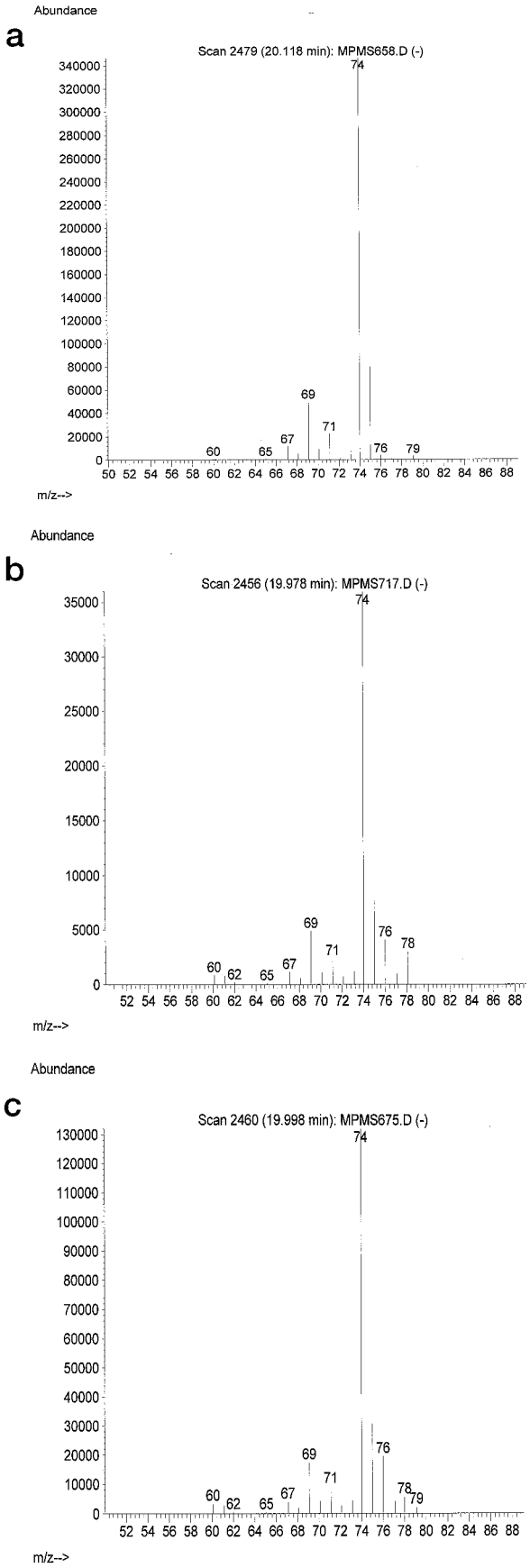

Figure 2. Mass spectral analysis (McLafferty region) of methyl oleate, after hydrogenation, from a 3-h incubation of [$^{13}C_2$,$^{18}O_2$]acetic acid with spinach leaf tissue. The figure shows the unlabeled control (a), oleate derived from MGDG (b), and oleate derived from PC (c).

equilibrium with carbon dioxide and water, aldolase and transaldolase reactions (Model et al., 1968), the rapid equilibration of the keto group oxygen in dihydroxyacetone phosphate with water (Model et al., 1968), and exchange of the keto group oxygen in pyruvate with water mediated by chemical hydration-dehydration and through the interconversion of pyruvate and Ala by transaminases. Thus, there was a potential problem in using [^{18}O]water as a substrate in that the carboxyl oxygen of fatty acids could be labeled at or close to the ^{18}O content of the water prior to any fatty acyl hydrolysis and re-esterification reaction. In such a case a hydrolysis reaction would not be easily detected or quantified.

To avoid such pitfalls, the experimental strategy shown in Figure 3 was used. The carbon source for fatty acid synthesis in chloroplasts in vivo has been the topic of considerable debate. Suffice it to say for the purposes of this work that acetate appears to be a very effective substrate for fatty acid synthesis not only in isolated chloroplasts (Roughan et al., 1978, 1979) but also with leaf tissue (Slack and Roughan, 1975). With [$^{13}C_2{}^{18}O_2$]acetic acid as a substrate, [^{18}O]fatty acyl thioesters are first produced and subsequent hydrolytic reactions can be examined by loss of ^{18}O from the fatty acyl groups. When an ester or thioester is hydrolyzed, an oxygen atom from water is introduced into the free fatty acid product. Since the carboxylate anion is symmetrical and because rates of protonation and deprotonation are very fast, the oxygen atom derived from water very rapidly becomes equivalent to the oxygen derived from the ester or thioester. Either oxygen may then become the carbonyl oxygen in a subsequent acyl transfer reaction. Thus, after synthesis of the fatty acid every hydrolytic cycle would result in a 50% loss of ^{18}O content. In addition, at the same time that ^{18}O labeling is occurring for the purpose of tracking the fate of the carbonyl oxygen atoms the newly synthesized fatty acids are additionally labeled with ^{13}C from acetate and so can be readily distinguished from endogenous fatty acids by GC-MS. This concurrent ^{13}C labeling allows the absolute ^{18}O content to be measured, and it is also economical with ^{18}O, which is very expensive.

Acetate Incorporation: Concentration Curve, Time Course, and Products

For acetate labeling to be effective, the first issue was to determine if enough acetate could be utilized for fatty acid synthesis in fairly short assay periods to yield a good signal for newly synthesized fatty acids using MS. Figure 4 shows the rate of acetate incorporation into fatty acids as a function of the acetate concentration in the medium, up to 20 mM, and at pH 5.7. The maximal rate occurred at 15 mM, with a half-maximal rate at 2 mM. The maximal rate for this experiment, in which slightly older spinach plants and

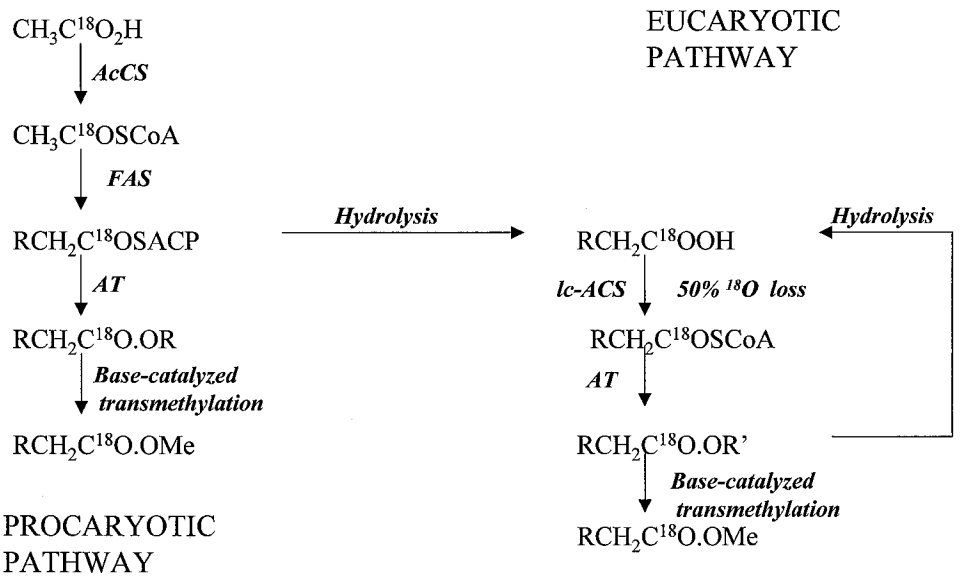

Figure 3. Rationale for the [$^{13}C_2$,$^{18}O_2$]acetate dual labeling experiment. Abbreviations for enzymatic steps are: AcCS, acetyl-CoA synthetase; lc-ACS, long-chain acyl-CoA synthetase; FAS, ACP-dependent fatty acid synthesis; and AT, acyltransferase (to ROH or R'OH as acyl acceptor).

leaves were used, was 0.5 μmol h^{-1} mg^{-1} chlorophyll. The major labeled lipid classes were monogalactosyldiglyceride (28%–34%) and phosphatidylcholine (27%–34%), the values being entirely independent of the acetate concentration. At the end of the 3-h assay, labeled total fatty acids were saturates (22%–31%, largely palmitate), monoenes (35%–45%, largely oleate), dienes (17%–22%, largely linoleate), and trienes (13%–17%, largely α-linolenate). Again, there was no correlation between acetate concentration and percentage label. These lipid and fatty acid product distributions are consistent with those reported in many studies for acetate labeling of leaves from a variety of species, as summarized by Roughan and Slack (1982). We can conclude from this experiment that the high acetate concentrations required to sustain high rates of fatty acid synthesis in intact leaf tissues do not perturb the distribution of lipid and fatty acid products compared with tracer labeling. Acetate concentrations in the 2 to 10 mM range were used for further experiments. Figure 4 also shows the time course for acetate incorporation into fatty acids in the leaf strip assay. Over a 5-h period there was a linear incorporation.

Mass Spectral Analysis for Assays with [1-^{13}C]Acetate

Assays with [1-^{13}C]acetic acid were undertaken first and the mass spectra for FAME from total lipids were examined. The molecular ion region for oleate isolated from leaf strips incubated with 10 mM [1-^{13}C]acetate for 2 h (Fig. 5a) shows a cluster of peaks with greater *m/z* values than the natural abundance control spectrum. This indicates incorporation of the ^{13}C label. Summing these peaks after subtracting the natural abundance background, and weighting each peak for the number of ^{13}C atoms it contains, gives a 11.5% ^{13}C content (over natural abundance) for the oleate peak. Likewise, an analysis of the McLafferty peak region for the same sample gave 11.9% ^{13}C content (over natural abundance) for the oleate peak, based on a comparison of the intensities of the peaks at m/z = 74 and 75. These ^{13}C content values can be accurately measured down to values of $\pm$0.2%. The correspondence in the values for ^{13}C content obtained by analysis of the molecular ion cluster (11.5%) and the McLafferty peak (11.9%), in which just C(1) and C(2) of the fatty acyl chain are examined, suggest that the distributions of exogenous acetate along the acyl chain are essentially random. Because [1-^{13}C]acetate was used as substrate, an average value of 11.7% for ^{13}C content translates to a 23.4% utilization of exogenous acetate in total oleate carbon atoms. Total oleate is comprised of both the pre-exisitng oleate pool and the "newly-synthesized" oleate of the assay period.

In Figure 5a it is possible to discern a population of oleate molecules where all nine C2 units are supplied by exogenous acetate (m/z = 305). By subtracting the natural abundance molecular ion distribution from the labeled oleate distribution it is possible to obtain the $^{13}C_n$ distribution resulting from the incorporation of exogenous acetate (Fig. 5b). When obtaining these $^{13}C_n$ distributions, only the contribution of $^{13}C_0$ to de novo fatty acid synthesis during the period of the assay cannot be determined directly, since it is masked by the much greater mass of the natural abundance molecular ion peak of the fatty acid prior to the incubation. However, at 10 mM [1-^{13}C]acetate, extrapolating to obtain a value for the newly synthesized $^{13}C_0$ species, we obtained a value of 46% of the total carbon in the newly synthesized oleic acid originates from exogenous acetate. Similar results to those shown for labeled oleate in Figure 5 were obtained for labeled palmitate (data not shown). Thus, even at near-saturating concentrations of

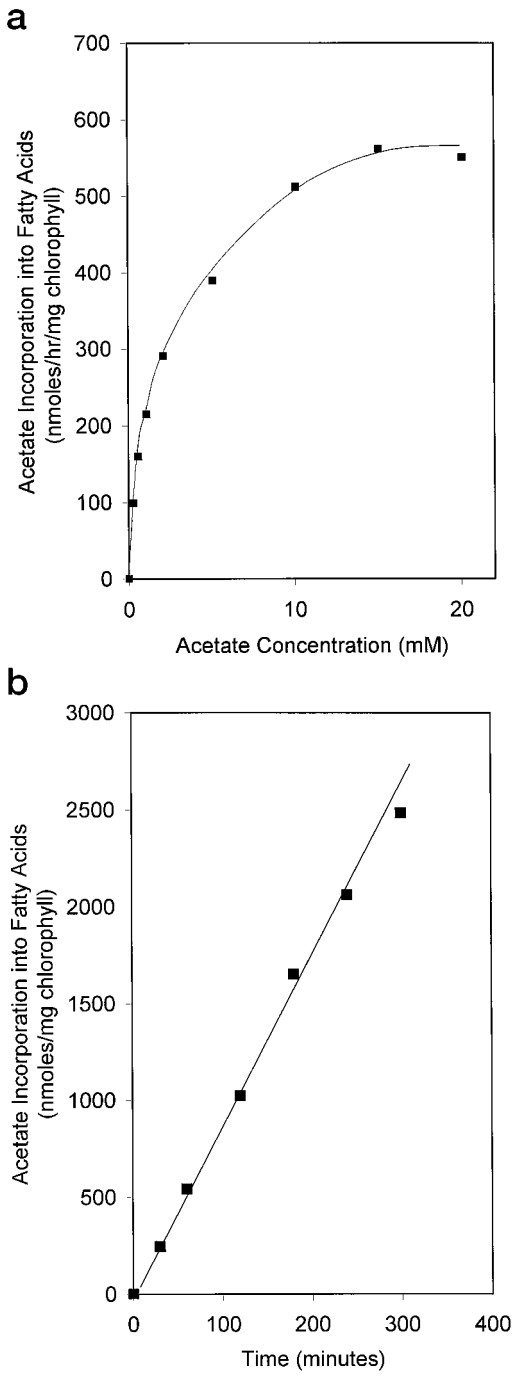

Figure 4. a, Concentration curve for acetate incorporation into total fatty acids in spinach leaf assays. b, Time course for acetate incorporation into total fatty acids in spinach leaf assays. The time course was run at 2 mM acetate, and gave a rate of 520 nmol acetate h^{-1} mg^{-1} chlorophyll. Data points are averages of two determinations.

exogenous acetate, endogenous carbon sources contribute about one-half of the substrate for fatty acid synthesis.

Mass Spectral Analysis of Assays with [$^{13}C_2$$^{18}O_2$]Acetate

Since labeling of spinach leaf fatty acids, and particularly palmitate and oleate, at millimolar concentrations of [1-^{13}C]acetate results in stable isotope accumulation that is easily detectable and accurately quantitated by GC-MS, [$^{13}C_2$,$^{18}O_2$]acetate was prepared for subsequent experiments on the nature of the putative hydrolytic step. The experimental design is shown in Figure 3, based on the "two-pathway" hypothesis for the fatty acid and lipid synthesis in the plant cell. Oleate and palmitate, and linoleate (plus hexadecenoate) from incubations of spinach leaf strips with [$^{13}C_2$,$^{18}O_2$]acetate were isolated from MGDG and phosphatidylcholine (PC), respectively, hydrogenated,

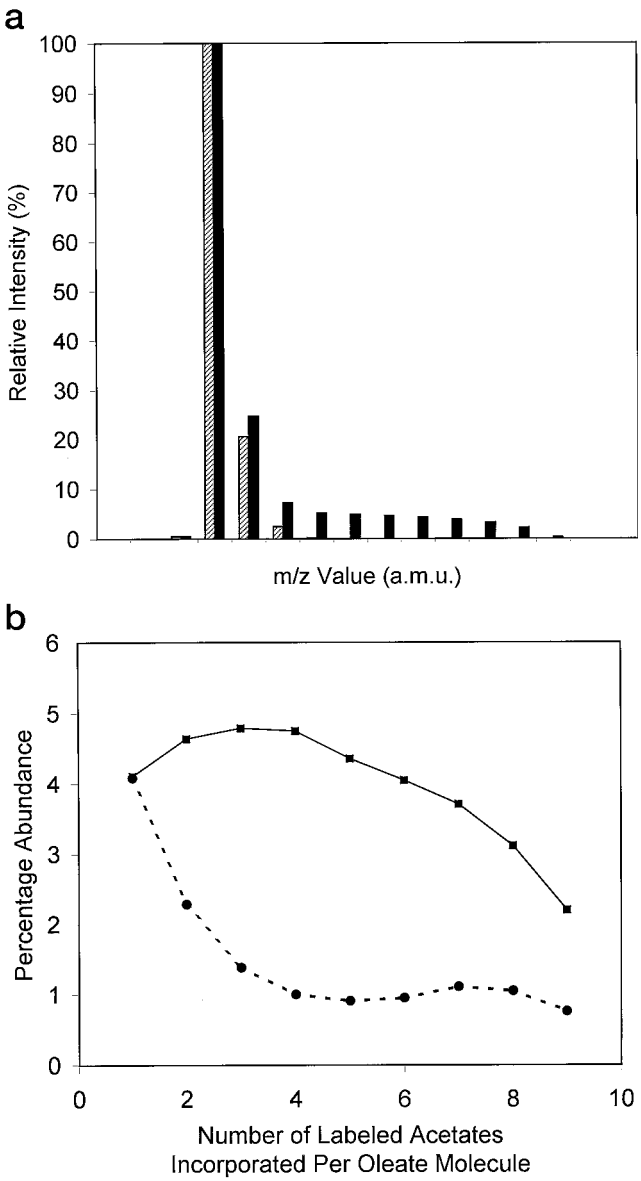

Figure 5. a, Molecular ion cluster for methyl oleate labeled with 10 mM [1-^{13}C]acetate for 2 h compared with the control. The base peak (100%) at *m/z* = 296 represents methyl oleate contining ^{12}C, ^{1}H, and ^{16}O isotopes only. Hatched bars, Unlabeled oleate; solid bars, oleate from [1-^{13}C]acetate labeling, with each bar representing an increment of one atomic mass unit (a.m.u.). b, The $^{13}C_n$ distribution for methyl oleate resulting from the incorporation of exogenous [1-^{13}C]acetate at both 1 mM (●) and 10 mM (■) concentrations.

and analyzed by GC-MS. Representative mass spectra for the McLafferty region, which is used for the biosynthetic ^{18}O content measurement, are shown in Figure 2. Inspection of this figure clearly shows that the ratio of $m/z = 78$ to $m/z = 76$ peaks decreases in the fatty acid isolated from PC compared with MGDG.

The percentage ^{18}O content for FAME isolated from MGDG and PC were plotted as a function of time of assay in Figure 6. In addition, the [$^{13}C_2$,$^{18}O_2$]acetate in the medium was directly analyzed by GC-MS to assess its ^{18}O content over time. These control data included in Figure 6 clearly show that the ^{18}O content of the acetic acid in the medium did not change over the course of the incubation, but remained constant at 57%. C16 fatty acids isolated from the prokaryotic lipid MGDG showed a small reduction in ^{18}O content, to 53%, which barely decreased with assay time. C18 fatty acids isolated from the prokaryotic lipid MGDG showed an ^{18}O content at zero time of about 53%, but this value decreased significantly over the 3-h assay period. Fatty acids isolated from the eukaryotic lipid PC showed a large reduction in ^{18}O content compared with the substrate and fatty acids in prokaryotic lipids; that is, a 25% ^{18}O content when extrapolated to zero time. The reduction in this value over time was small, with palmitate and oleate declining to 23.5% in the 3-h assay, while linoleate declined to 21.5%, a value that is statistically significantly lower than for palmitate or oleate.

Molecular species of MGDG from the 3-h incubation (four pooled assays) were analyzed for the ^{18}O content of their fatty acids. The MGDG fraction isolated by preparative silica TLC was further fractionated into molecular species by C_{18} RP-TLC, and six labeled bands were observed ranging from the least mobile molecular species, 16:0/18:1, to the most mobile, 16:3/18:3. The two bands with the greatest R_F value and comprising most of the endogenous mass were discarded (16:3/18:3 and 18:3/18:3 plus 16:2/18:3). In the 3-h assay period much of the label was present as 16:0, 16:1, 18:1, and 18:2 fatty acids, rather than 16:3 and 18:3. However, the predominant endogenous fatty acids were 16:3 and 18:3. Thus, removal of 16:3/18:3, 18:3/18:3, and 16:2/18:3 molecular species allows a significant enrichment of the label in the remaining C16 and C18 fatty acids, facilitating the analysis described below. The four bands comprising the less-unsaturated molecular species were pooled, hydrogenated, and separated into C16/C18 and C18/C18 molecular species by C_{18} RP-TLC. The hydrogenated MGDG species were transmethylated and the FAME analyzed by GC-MS. C16 FAME from the C16/C18 MGDG molecular species had an ^{18}O content of 54%; C18 FAME from the C16/C18 MGDG molecular species had an ^{18}O content of 55%; and C18 FAME from the C18/C18 MGDG molecular species had an ^{18}O content of 27%.

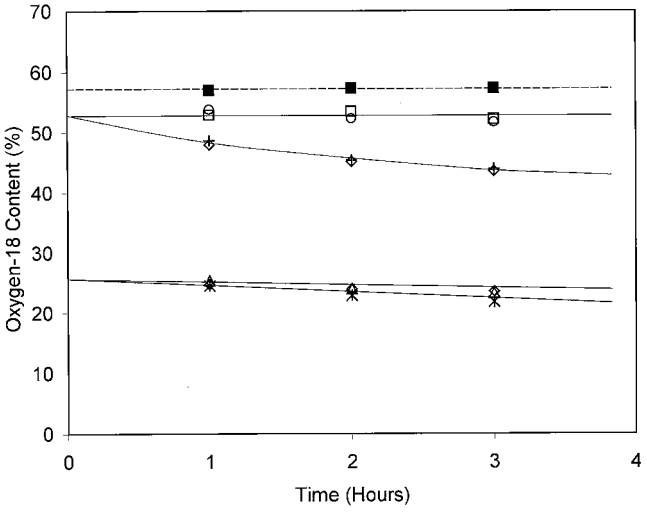

Figure 6. ^{18}O content of isolated fatty acids from [$^{13}C_2{}^{18}O_2$]acetic acid incubation with spinach leaf tissue. Each data point represents the average of duplicate GC-MS determinations for each of three independent assays. Error bars for ranges are not shown, but range from a maximum of 3.7% ^{18}O content for 16:0-MGDG at 1 h to a minimum of 0.4% ^{18}O content for 18:2-PC at 3 h. Ordered from the highest ^{18}O content to the lowest at 3 h are acetate (■), 16:0 in MGDG (□), 16:1 in MGDG (○), 18:1 in MGDG (+), 18:2 in MGDG (◇), 18:1 in PC (◇), 16:0 in PC (△), and 18:2 in PC (*).

DISCUSSION

Exogenous Acetate Can Sustain High Rates of Fatty Acid Synthesis in Leaf Tissues

A combination of [^{14}C] and [^{13}C]acetate labeling in the same assay can be used to estimate total rates of fatty acid synthesis in leaf tissues. The radiolabel incorporated into total fatty acids and the specific activity of the acetate give the exogenous rate, while the isotopic distributions of the molecular ion peak give relative contributions of exogenous and endogenous carbon sources. At a 10 mM acetate concentration, which is approaching saturation, the rate of exogenous acetate incorporation ranged between 0.5 and 2.0 μmol acetate h^{-1} mg^{-1} chlorophyll, depending on the age of the spinach leaves used (with younger leaves giving higher rates). The $^{13}C_n$ distributions in oleate and palmitate labeled with [1-^{13}C]acetate indicate that at a 10 mM concentration, about 40% to 50% of the C2 units were derived from exogenous acetate, such that the endogenous contribution was also 0.5 to 2 μmol h^{-1} mg^{-1} chlorophyll and the combined activities were 1 to 4 μmol C2 units h^{-1} mg^{-1} chlorophyll. These activities are in line with estimates of the maximum rates of fatty acids synthesis in isolated chloroplasts of 1 to 2 μmol C2 units h^{-1} mg^{-1} chlorophyll (Roughan, 1987), with the use of [^3H]water to label fatty acids in leaf discs, when a rate equivalent to 1.28 μmol C2 units h^{-1} mg^{-1} chlorophyll was estimated (Browse et al., 1981), and with [^{13}C]carbon dioxide labeling studies of Arabidopsis leaves undertaken in our laboratory (X. Bao, M. Focke, M. Pollard, and J. Ohlrogge, unpublished data) in which a rate of 1.14 μmol C2 units h^{-1} mg^{-1} chlorophyll was determined.

The observed molecular ion $^{13}C_n$ distributions for fatty acids labeled with [1-^{13}C]acetate in leaf tissue (Fig. 5b) did not in any way resemble simple theoretical distributions. At the lower acetate concentration (1 mM) shown in Figure 5b it is possible to discern at least two components to the molecular ion $^{13}C_n$ distribution. The observed distributions

are similar for young and old leaves (data not shown). With the leaf strips used in the assays these heterogeneous distributions may arise from contributions from cells of different types and at different stages of development with different capacities to synthesize fatty acids from acetate. The heterogeneous distributions may also arise from different acetate transport processes into the tissue, since acetate import may occur through stomata, through cut veins, and through the cut surfaces of the bulk mesophyll tissue. Whatever the reason, acetate utilization by leaf fatty acid synthesis is very effective; what is most important is that the distributions suggest that the endogenous substrate pool of acetyl-CoA for fatty acid synthesis is mixing with the pool of acetyl-CoA derived from exogenous acetate. This inference is important, because it implies that the conclusions we draw below from exogenous [$^{13}C_2$,$^{18}O_2$]acetate labeling concerning the export of fatty acids from the chloroplast applies equally to fatty acids synthesized from endogenous substrate.

Prokaryotic Lipid Synthesis Proceeds without a Hydrolytic Step after Fatty Acid Synthesis

The labeling from [$^{13}C_2$,$^{18}O_2$]acetic acid very clearly shows that there is a minimal reduction (from 57% to 53%) in ^{18}O content for the fatty acids in the prokaryotic lipids compared with the acetic acid substrate. This is expected if the acetic acid undergoes only a single activation step in diffusing from the medium and being converted to acetyl-CoA at the site of fatty acid synthesis, the chloroplast. This confirms that the prokaryotic lipids are indeed synthesized by direct acyl transfer from acyl-ACP to acyl acceptor. Since both palmitate (*sn*-2) and oleate (*sn*-1) show a similar ^{18}O content at zero time, this conclusion applies to both acyl transferases.

Eukaryotic Lipid Synthesis Proceeds with a Hydrolytic Step after Fatty Acid Synthesis

Fatty acids isolated from phosphatidylcholine showed an approximately 50% loss of ^{18}O content (from 53% to 25% when extrapolated to time zero) compared with fatty acids isolated from MGDG. This confirms that most if not all of the fatty acyl groups exported from the plastid must be undergoing a single hydrolysis reaction. If there was a direct thioester transfer, it is likely that there would be 100% conservation of ^{18}O. We do not have a plausible enzyme mechanism to describe opposite situation, the complete loss of ^{18}O, but simply note this theoretical possibility from the point of view that enzymes generally are completely stereospecific in their underlying chemistry. The results rule out the conservation of the thioester bond during acyl export from the plastid, either directly (even if driven by ATP-dependent process to make it directional) or by a "high-energy" intermediate such as acyl-carnitine. An argument has been made that the conservation of the energy of the thioester bond would be preferable during fatty acid export (Thomas et al., 1982, 1983; McLaren et al., 1985). Countering this argument, it should be noted that the expenditure of an additional ATP in the synthesis of a C18 fatty acid that requires on a molar basis 8 mol of ATP and 16 mol of NAD(P)H for synthesis from acetyl-CoA is not great. Also, it seems logical that an acyl exchange reaction between ACP and CoA, which might be close to unity in terms of thermodynamic equilibrium, is not an ideal way to export fatty acyl groups unidirectionally from an organelle.

Distinct Prokaryotic and Eukaryotic MGDG Species Are Defined by ^{18}O Labeling

Spinach leaves contain about 50% *sn*-1 C18, *sn*-2 C16 (prokaryotic) and 50% *sn*-1 C18, *sn*-2 C18 (eukaryotic) species of total galactolipids, with only "traces" of other species (Roughan and Slack, 1984). The ^{18}O content for C16 fatty acids in total MGDG shows only a very small decline with time, but in contrast the ^{18}O content for C18 fatty acids in total MGDG declines significantly (Fig. 6), presumably due to a eukaryotic contribution. This was confirmed by the analysis of the MGDG molecular species. Both C16 and C18 fatty acids in the C16/C18 molecular species had ^{18}O contents indicating no hydrolysis after fatty acid synthesis (54%–55%), while the C18 fatty acids in the C18/C18 molecular species had ^{18}O contents indicating a single hydrolytic step after fatty acid synthesis (27%). Thus, the biosynthesis of MGDG in leaf tissue in "16:3" plants postulated to occur via the prokaryotic pathway for C16/C18 molecular species (Roughan and Slack, 1982, 1984; Somerville and Browse, 1991), but also by the eukaryotic pathway, involving reimport of cytosolic lipid components, for di-C18 molecular species of MGDG, has been confirmed by this ^{18}O-labeling experiment.

Kinetic Isotope Effects

The conversion of acetic acid to fatty acids in prokaryotic lipids gives a reduction in ^{18}O content of 57% to 53%, whereas the hydrolytic export of fatty acids from the plastid represents a reduction of 53% to 25%. For the acetic acid to fatty acid conversion, if acetyl-CoA did not undergo any hydrolysis and re-activation, there should not be a reduction at all, while for the export of fatty acids from the plastid the reduction should be 50% (i.e. from 53% to 26.5%). These expectations are predicated on a negligible kinetic isotope effect. We are unaware of data giving the magnitude and direction of ^{18}O kinetic isotope effects for activation of carboxylic acids, but note that maximum theoretical values for primary kinetic isotope effects involving ^{18}O are on the order of 1.05 to 1.07, while maximum observed values for primary kinetic isotope effects involving ^{18}O in ester hydrolysis are 1.06 and in water ionization/protonation reactions 0.96 to 1.02 (Shiner and Wilgis, 1992). We speculate that the slight discrimination against ^{18}O that we observe might be caused in part by a small kinetic isotope effect for the activation of carboxylic acids to CoA thioesters.

Acyl Turnover in Phospholipids

Figure 6 shows that over the 3-h time period there was a small loss of ^{18}O content in fatty acids in PC, indicating

slow acyl turnover via a hydrolytic mechanism. The reduction in ^{18}O content was from 25% to 23.5% for palmitate and oleate, and from 25% to 21.5% for linoleate. The decline for linoleic acid, of 3.5%, is certainly statistically valid, since at 3 h the SD for multiple determinations of ^{18}O content in FAME is $<\pm 0.5\%$. Since the [$^{13}C_2{}^{18}O_2$]acetic acid substrate in the medium did not show a decline in its ^{18}O content value over time (Fig. 6), and since the decline in ^{18}O content in C16 fatty acids in MGDG over 3 h was <1% (Fig. 6), most of the decline in the ^{18}O content of linoleate in PC can be attributed to hydrolytic turnover.

These small declines in percentage ^{18}O content belie a much greater turnover time for two reasons. First, a single hydrolytic turnover results in a 50% reduction in the ^{18}O content (Fig. 3). This means that a single hydrolytic turnover of linoleate would result in a decline of ^{18}O content from 25% to 12.5%. And second, since labeling is continuous and essentially linear, the average residence time for a fatty acid in PC is only 1.5 h. Thus, a 3.5% drop in ^{18}O content in linoleate in PC over 3 h translates to approximately a 18% linoleoyl turnover per hour. Further experiments on acyl turnover in phospholipids are under way in our laboratory, and we plan to address the issue of whether such a turnover in leaf tissue is constitutive or a result of a wound response from preparing leaf strips for assay.

The Nature of Fatty Acyl Export from the Chloroplast

The results of this study indicate that the export of fatty acyl groups from the chloroplast requires a free fatty acid; that is, the carboxylate oxygen atoms have time to become equivalent. However, the free fatty acid transfer mechanism remains unknown. The acyl group has to traverse the inner envelope membrane and the periplasmic space between the chloroplast envelopes to reach the outer envelope, since the inner and outer envelopes appear not to be contiguous (although there appear to be "contact zones") (Douce and Joyard, 1990). Also, as we do not know the vectorial nature of the acyl-CoA synthetase reaction in the outer envelope, the acyl group may or may not have to traverse the outer envelope bilayer as well. Thus, the mechanism for acyl export from the chloroplast may be quite complex, and could even involve acyl binding proteins from the cytosol, should they penetrate the periplasmic space. At one end of the spectrum of possibilities, free fatty acid diffusion from the chloroplast could be considered. At the other end, a channeled active transport mechanism could be postulated. These represent interesting questions for further studies, which are ongoing in our laboratory.

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