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Spinach Leaves Desaturate Exogenous [^{14}C]Palmitate to Hexadecatrienoate[1]

EVIDENCE THAT *DE NOVO* GLYCEROLIPID SYNTHESIS IN CHLOROPLASTS CAN UTILIZE FREE FATTY ACIDS IMPORTED FROM OTHER CELLULAR COMPARTMENTS

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ABSTRACT

Long-chain ^{14}C-fatty acids applied to the surface of expanding spinach leaves were incorporated into all major lipid classes. When applied in diethyleneglycol monomethyl ether solution, as done by previous workers, [^{14}C]palmitic acid uptake was much lower than that of [^{14}C] oleic acid. However, when applied in a thin film of liquid paraffin the rate of [^{14}C] palmitic acid metabolism was rapid and virtually complete. Considerable radioactivity from [^{14}C]palmitate incorporated into lipids following either application method gradually appeared in polyunsaturated C_{16} fatty acids esterified to those molecular species of galactolipids previously thought to be made using only fatty acids synthesized and retained within the chloroplast. Evidence for the incorporation of radioactivity from exogenous [^{14}C]oleate into those same molecular species of galactolipids was less compelling. The unexpected availability of fatty acids bound to extrachloroplastidal lipids for incorporation into galactolipids characteristically assembled entirely within the chloroplast emphasizes the need to reassess interrelations between the "prokaryotic" and "eukaryotic" pathways of galactolipid formation.

Fatty acid (FA)[3] synthesis in plant cells occurs entirely within the chloroplast. The products of this synthesis, mainly palmitic (16:0) and oleic (18:1) acids, are utilized by two distinct metabolic pathways for the formation of membrane glycerolipids (25). One sequence of reactions, confined to the chloroplast, appears to utilize *de novo* synthesized fatty acyl-ACP for the formation of phosphatidic acid, phosphatidylglycerol (PG) and, in some species, glycolipids (11). Because the specificities of these plastid enzymes closely resemble those of equivalent cyanobacterial enzymes, the term prokaryotic pathway is applied to this scheme.

In contrast, an appreciable and in some species a major proportion of the cell's FA is transported out of the chloroplast for incorporation into phospholipids by extrachloroplastidal enzymes. These microsomal enzymes have markedly different specificities for acylation and constitute the eukaryotic pathway. Much of the phosphatidylcholine (PC) formed in this fashion is subsequently utilized as a donor of modified acyl chains, probably in the form of diacylglycerol, for reimportation into the chloroplast and incorporation into eukaryotic type glycolipids there. Schemes devised to explain the observed kinetics of cytoplasmic FA movement into the chloroplast make no provision for the entry of these FA into lipids of the prokaryotic type (9, 25).

It was therefore surprising to find that exogenous ^{14}C-FA administered to the green alga *Dunaliella salina* were able not only to enter the lipids of cytoplasmic membranes but also readily move into the chloroplast for utilization by the prokaryotic pathway (21). This observation provided a logical explanation for an earlier report (13) that photoautotrophically grown *Chlorella vulgaris* cells could desaturate exogenous 16:0 to FA characteristically found in prokaryotic lipids. However, leaf tissues from higher plants, although able to incorporate and desaturate [^{14}C]18:1 (14, 16, 20, 23, 28) and elongate and desaturate [^{14}C] 12:0 (2, 15, 17), appeared incapable of modifying [^{14}C]16:0 following its entry into cells (2, 15, 17). In retrospect, this inability to observe any metabolic alteration of exogenous 16:0 may have resulted from a fortuitous selection of higher plant material; all of the plants supplied with exogenous [^{14}C]16:0 to date have been 18:3-plants, so designated because they lack the 16:3-containing prokaryotic lipids (25). It is only 16:3-plants that offer the opportunity for 16:0 desaturation through to 16:3. The experiments described below examine the fate of exogenous [^{14}C] FA in the 16:3-plant *Spinacia oleracea*.

MATERIALS AND METHODS

Spinacia oleracea (Yates hybrid 7 and hybrid 102) plants were grown in an aerated nutrient solution as described previously (27) and exposed to 500 μE/min·m^2 of light during a 10 h/14 h light/dark cycle. *Arabidopsis thaliana* was grown in continuous light (200 μE/min·m^2) in a 25°C growth chamber. *Pisum sativum* was grown in the greenhouse under natural light.

1-[^{14}C]Palmitic acid (61 mCi/mmol), 1-[^{14}C]oleic acid (56.7 mCi/mmol), 1[^{14}C]lauric acid (26 mCi/mmol), and sodium 1-[^{14}C]acetate (58 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, and liquid paraffin (mineral oil) (0.870–0.890 g/ml density) from May and Baker Ltd., Dagenham, England.

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[3] Abbreviations: FA, fatty acid; GL, glycolipids; PC, phosphatidylcholine; MGD, monogalactosyldiacylglycerol; DGD, digalactosyldiacylglycerol; PG, phosphatidylglycerol; SL, sulfolipid; FAME, fatty acid methyl ester; DGME, diethyleneglycol monomethyl ether.

Unless otherwise stated, ^{14}C-FA were applied to the upper surface of expanding leaves in 0.2 to 0.5 μl droplets of diethyleneglycol monomethyl ether (DGME) (BDH Chemicals Ltd., Poole, England) so that each leaf received 5 to 10 μl solvent (0.5–1.0 μCi ^{14}C-FA). The spinach and pea leaves utilized were 2 to 4 cm long while *Arabidopsis* leaves were 0.2 to 1.5 cm long. Control leaves showed no visible signs of damage over a several day period when twice the normal amount of DGME was applied. In control experiments, the percentage of lipid radioactivity found in PG, monogalactosyldiacylglycerol (MGD), and digalactosyldiacylglycerol (DGD) after [^{14}C]acetate was applied to leaves in DGME exceeded only slightly that found after [^{14}C]acetate application in water, indicating that an effect of a DGME-related solvent on lipid metabolism noted by Gardiner *et al.* (12) was not leading to serious artifacts in our experiments utilizing DGME.

In some experiments an aliquot of the ^{14}C-FA in toluene solution was evaporated on the 0.5 × 0.3 cm tip of a stainless steel spatula and applied to the leaf surface either directly by gentle rubbing or after additionally evaporating on the spatula tip 5 μl petroleum ether containing 0.5 mg liquid paraffin. No damage or growth inhibition was evident over a period of several days even when 10 times more liquid paraffin was spread over the upper surface of control spinach leaves.

In most experiments leaf tissue was steamed for 10 min to inactivate any lipolytic enzymes present and then lipids were extracted by the procedure of Bligh and Dyer (6). As a control, some leaves were quickly frozen in liquid N_2 before extracting lipids by the procedure of Browse *et al.* (7). Equivalent results were obtained by the two extraction procedures. Recovery of radioactivity in lipids following ^{14}C-FA labeling was nearly complete. The aqueous residue following lipid extraction contained <2% of the recovered ^{14}C, indicating little if any ^{14}C-FA degradation to water-soluble components.

Individual lipid classes were separated from the extracts by two dimensional TLC on silica gel G with development first in chloroform:methanol:13M NH_4OH (13:5:0.5)[4] and then in chloroform:methanol:glacial acetic acid:water (20:3.5:2.3:0.7). Lipid spots were visualized in I_2 vapors and scraped off for scintillation counting. MGD and DGD were purified on 20 cm wide silica gel G TLC plates as follows. Total lipid extracts were chromatographed first in acetone:benzene (3:2) to move waxes and pigments to the top of the plate and MGD to an R_F of 0.6. The MGD band was visualized under UV light after spraying the upper part of the plate with dichlorofluorescein solution and was recovered from the silica gel scrapings by adding in sequence 5 ml acetone, 4 ml H_2O, and 5 ml petroleum ether, shaking vigorously and centrifuging. The petroleum ether layer was recovered, the aqueous methanol was reextracted with a further 2.5 ml of petroleum ether, and the combined petroleum ether extracts were concentrated under N_2. Immediately after removing the MGD band the lower part of the plate was rechromatographed in acetone:benzene:H_2O (18:6:0.8), which caused DGD to move to an R_F of 0.6. DGD was also visualized with dichlorofluorescein and was extracted from the scrapings by adding 0.5 ml H_2O, 2.5 ml methanol, and 2.5 ml chloroform, in sequence, and then mixing and centrifuging. After reextracting the silica gel with the same volumes of the above solvents, the combined extracts were washed with 3.5 ml 1% NaCl (containing 1 drop NH_4OH), and the lower phases were recovered and concentrated.

Fatty acid methyl esters were resolved on 20 × 20 cm silica gel G TLC plates activated at 110°C for 30 min and prerun in petroleum ether (60–80°C) containing 5% liquid paraffin. After spotting samples and reference standard FAME (20:1 to locate 18:0; 18:1 for 18:1 and 16:0; 18:2 for 18:2 and 16:1; 18:3 for 18:3 and 16:2; and 16:3) the plate was developed in acetonitrile:methanol:H_2O (6:3:1) saturated with liquid paraffin, and bands were visualized in I_2 vapors. Radioactivity was measured by autoradiography or scintillation counting of scraped bands. In some cases, FAME were resolved by argentation TLC by the procedure of Morris (19). Neutral glycerides were separated by TLC using petroleum ether:ethyl ether:acetic acid (70:30:1).

FAs were hydrolyzed from galactolipids using a lipase preparation from *Rhizopus arrhizus* (Serva, Heidelburg, West Germany, 58 units/mg). Lipids were dissolved in a mixture of 0.5 ml H_2O-saturated ethyl ether, 0.2 ml 0.1 M borate buffer (pH 7.4), and 10 μl (5.8 units) lipase suspension and were incubated at 32°C for 2 h unless otherwise stated. After evaporating the ether with N_2, the hydrolysis products were extracted by adding 1.25 ml chloroform, 1.25 ml methanol, and 1 ml 1 M KCl in 0.2 M H_3PO_4 and recovering the lower phase. The products and unhydrolyzed substrate were resolved by TLC using chloroform:methanol:acetic acid (9:2:0.1).

Gas chromatography of FAME was accomplished using a 4 m × 3 mm column of ethyleneglycol succinate at 180–200°C. Hydrogenation at FAME were carried out with Adams catalyst by the procedure of Appelqvist (1). Surface wax was removed from spinach leaves by dipping them in cold hexane for 45 s (29). Leaves were then dipped in cold $CHCl_3$ for 45 to 60 s (4).

RESULTS

Incorporation of [^{14}C]Palmitic Acid by Spinach Leaves. Tracer amounts (0.5 μCi/9 nmol) of 1-[^{14}C]palmitic acid ([^{14}C]16:0) were dissolved in DGME, one of the two closely related solvents that have been widely used for applying ^{14}C-FA to plant tissue (16, 20, 23), and distributed as microdroplets on the upper surface of 5 to 8 d old spinach leaves that were doubling in fresh weight in approximately 4 d. The leaves were harvested for lipid extraction after 4 h or 1, 2, 4, 7, or 13 d. The initial esterification of [^{14}C]16:0 into glycerolipids was significant, with somewhat more than 20% being incorporated within 4 h, but utilization of the remaining free FA was extremely slow (Fig. 1, inset).

Radioactivity appeared in all the major phospholipid and glycolipid classes, as monitored by two dimensional TLC (Table I, column A). PC was the most highly labeled membrane component over relatively short time intervals. Its gradual decline in radioactivity and the corresponding rise in labeling of certain other lipids of interest are also illustrated in Figure 1. The percentages of radioactivity found in individual glycerolipids not

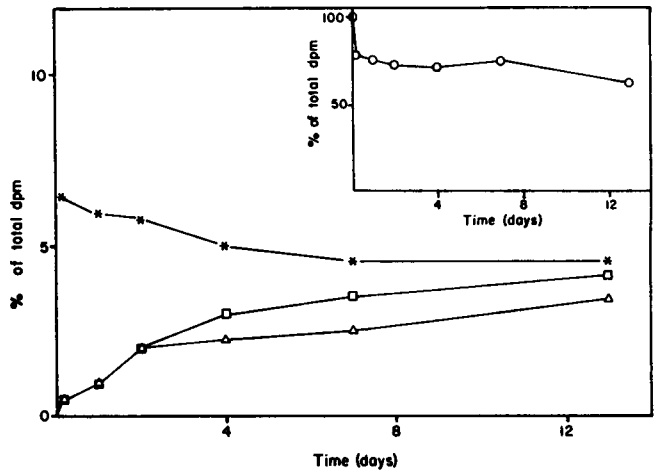

FIG. 1. Incorporation of [^{14}C]16:0 applied to a spinach leaf in DGME. Inset shows decline in free FA (O) radioactivity, while expanded scale indicates labeling of PC (*), MGD (Δ), and DGD (□). Points were calculated using lipid extracts having 830,000 ± 160,000 dpm/leaf.

[4] All solvents were mixed in volume proportions.

Table I. *Incorporation of ^{14}C-FA into Lipids of Spinach Leaves*

Each tracer was applied in DGME solvent, and lipids were extracted after 1 d. By this time the amount of radioactivity recovered from the TLC plate in ester-linked form was: column A, 16,750 dpm (23% of total leaf dpm); column B, 43,050 dpm (74% of total leaf dpm); column C, 18,546 dpm (94% of total leaf dpm). Radioactivity not in ester-linked form was present entirely as unincorporated ^{14}C-FA.

Lipid Class	Radioactivity		
	A [^{14}C]16:0 as tracer	B [^{14}C]18:1 as tracer	C [^{14}C]12:0 as tracer
	% of total dpm in ester-linked form		
Phosphatidylinositol	9	4	3
Phosphatidylcholine	25	45	16
Sulfolipid	3	1	7
Digalactosyldiacylglycerol	4	4	8
Phosphatidylglycerol	6	2	9
Phosphatidylethanolamine	17	12	7
Monoglactosyldiacylglycerol	4	16	19
Neutral lipids[a]	32	15	31

[a] Principally triacylglycerol.

plotted in Figure 1 remained relatively constant with time.

Spinach is designated a 16:3 plant, meaning that it contains in its chloroplast galactolipids some molecular species of the prokaryotic type, characterized by the presence of 16:1, 16:2, and 16:3 at the *sn*-2 position. C_{16}-FA, almost exclusively 16:0, might also be present in the *sn*-1 position of the eukaryotic glycolipid molecular species. The most prevalent glycolipid, MGD, was purified from the ^{14}C-labeled lipids by preparative TLC and analyzed to determine which FA had become labeled from exogenous [^{14}C]16:0.

Methyl esters were prepared from the MGD FA and these were separated by reverse phase TLC, sometimes in conjunction with argentation TLC. Radioactivity was found in bands representing trienoic, dienoic, and monoenoic FA as well as saturated FA (Table II). A gradual increase in labeling of the C_{16} polyunsaturates was noted with the passage of time. Autoradiography of one such TLC plate confirmed the absence of any closely associated bands of radioactive impurities; ^{14}C was uniformly distributed over the expected FAME compounds.

As indicated in Table II, most bands on the reverse phase TLC plate contained C_{18} as well as C_{16} FAME. Catalytic hydrogenation of total polar lipids from the leaf extracts followed by reverse phase TLC of FAME prepared from the hydrogenated products showed that there had been less than 7% elongation of the C_{16} FA to C_{18} FA during the labeling period. The authenticity of the major radioactive band, identified as 16:3, was confirmed by trapping the methyl ester of 16:3 from a gas chromatographic column and showing that radioactivity in the GC fraction was recovered in the 16:3 band when reanalyzed by reverse phase TLC.

Table II. *Distribution of Radioactivity among MGD FAME following Application of [^{14}C]Palmitic Acid to Spinach Leaves*

FAME prepared by treating purified MDG with $NaOCH_3$ were separated by reverse phase TLC.

Identity of Band	Incubation time with ^{14}C-16:0			Control ^{14}C-16:0 + Unlabeled MGD
	24 h	96 h	13 d	
	cpm (%)			
(18:0)[a]	87 (1)	58 (2)	83 (1)	181 (1)
16:0, (18:1)	1600 (23)	343 (10)	637 (8)	11860 (92)
16:1, (18:2)	1263 (18)	339 (10)	3238 (41)	338 (3)
16:2, (18:3)	1198 (17)	621 (19)		128 (1)
16:3	2225 (32)	1290 (39)	3711 (46)	97 (1)

[a] Fatty acids in parentheses known to contain virtually no radioactivity (see text).

According to the known distribution of FA in spinach MGD, the C_{16} polyunsaturates would be expected to occur at the *sn*-2 position (9). The positional distribution of radioactivity was determined by hydrolyzing purified MGD from [^{14}C]16:0-labeled leaves with the lipase from *Rhizopus arrhizus*, which has been reported (10) to remove the fatty acyl group specifically from the *sn*-1 position. In 6 analyses, involving leaves incubated with [^{14}C] 16:0 for periods ranging from 24 h to 13 d, the lyso-MGD, which still retained the *sn*-2-linked FA, had 10 to 36% of the total radioactivity of the hydrolysis products. Of the radioactivity recovered in lipase-released free FA, some was found in 16:3, and GC analysis of the released FA showed the presence of 2 to 4% 16:3 on a mass basis. It is not yet clear whether this 16:3 arose through acyl migration from the *sn*-2 position during the incubation with lipase or was, at least in part, situated at the *sn*-1 position *in vivo*.

Analysis of the purified DGD was also carried out by the techniques described above. Reverse phase TLC of purified DGD from 1, 4, and 13-d labeled leaves revealed that 17, 33, and 30%, respectively, of their total radioactivity was present in C_{16} unsaturates, mainly 16:2 and 16:3. In this case, too, *R. arrhizus* lipase analysis yielded lower than expected proportions of radioactivity in lyso-DGD, probably for the reasons mentioned above.

Incorporation of [^{14}C]Oleate by Spinach Leaves. In sharp contrast to the low incorporation of [^{14}C]16:0 into glycerolipids described above (Fig. 1), 1-[^{14}C]oleic acid ([^{14}C]18:1), when applied to young spinach leaves in the same manner, was readily and almost completely esterified into complex lipids. The added [^{14}C]18:1 radioactivity entered all major lipid classes (Table I, column B) and was slowly accumulated in MGD and DGD as PC and the residual free [^{14}C]18:1 declined (Fig. 2). There was no radioactivity in C_{16} FA, as determined by reverse phase TLC of hydrogenated FAME.

Spinach MGD contains two major molecular species, namely, 18:3/18:3 MGD, in which the diacylglycerol moiety is derived via the eukaryotic pathway, and 18:3/16:3 MGD, in which the diacylglycerol moiety arises through the prokaryotic pathway. MGD samples purified from leaves labeled for various time periods with [^{14}C]18:1 were resolved into these two principal classes by argentation TLC. After correcting for a small contamination of the slower moving 18:3/16:3 component by the 18:3/18:3 molecular species, radioactivity in the prokaryotic 18:3/16:3 species of MGD from leaves labeled for periods of 1 to 6 d

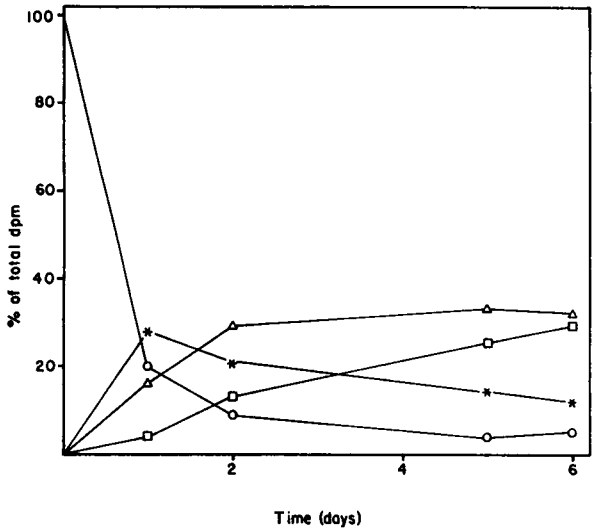

FIG. 2. Incorporation of [^{14}C]18:1 applied to a spinach leaf in DGME. Curves show changes in relative labeling of free FA (○), PC (*), MGD (△), and DGD (□). Points were calculated using lipid extracts having 514,000 ± 87,000 dpm/leaf.

contained an average of only 2.8% of the total MGD radioactivity. Considering that there are approximately equal proportions of these molecular species, *i.e.* 46% 18:3/16:3 and 52% 18:3/18:3 (5) and that the former molecular species has one C_{18} FA *versus* two in the latter, the specific radioactivity of 18:3 in the eukaryotic 18:3/18:3 component was calculated to be 15 times higher than that in the prokaryotic 18:3/16:3 species. Even this estimate of C_{18} FA labeling in prokaryotic MGD may be on the high side, since autoradiograms of argentation TLC plates upon which the 18:3/18:3 and 18:3/16:3 molecular species were resolved showed no sign of labeling in the latter component. This is in contrast to the active incorporation into prokaryotic MGD molecular species of FA synthesized *in situ* by spinach leaves (26).

In agreement with other workers (16, 20, 23) we found an almost inconsequential incorporation (1% of total lipid radioactivity after 1 to 6 d) of exogenous [^{14}C]18:1 into total leaf PG. This contribution is just one-tenth of that when 1-[^{14}C]acetate was the precursor (and one-third to one-seventh of that when [^{14}C]16:0 was the precursor), whereas the contributions to total lipid radioactivity by all of the other glycerolipids were similar with either [^{14}C]oleate or [^{14}C]acetate as the precursor (data not shown). This phenomenon may be even more significant if the PG that is labeled by exogenous [^{14}C]18:1 is extrachloroplastidal, thus reducing even further the likelihood that the labeled precursor entered the prokaryotic lipid in sizeable amounts.

Factors Affecting the Pattern of Exogenous FA Utilization. Several control experiments were carried out in an effort to understand the mechanism of exogenous ^{14}C-FA uptake and to explain the low proportion of [^{14}C]16:0 taken up into ester linkage in comparison with the incorporation of [^{14}C]18:1. A third radiotracer, [^{14}C]lauric acid ([^{14}C]12:0), when applied to expanding leaves, was rapidly utilized, with 94% of the administered label being incorporated into complex lipids (Table I, column C) after both 1 d and 4 d incubations. Analysis of polar lipid FAME by reverse phase TLC revealed that there had been extensive elongation and desaturation. Catalytic hydrogenation of FAME from 4 d labeling periods indicated 52 and 36% of the total radioactivity in C_{18} and C_{16} FA, respectively.

It was postulated that the more effective uptake of [^{14}C]18:1 and [^{14}C]12:0 might be due to the fact that the melting points of these FA are considerably lower than that of 16:0. In an effort to modify the physical state of [^{14}C]16:0, it was mixed with an equimolar amount of nonradioactive 18:1 in chloroform solution, and after evaporating the solvent, the mixture was redissolved in the standard application solvent, DGME, and applied to leaves. After 1 and 3 d incubations, the incorporation into glycerolipids of [^{14}C]16:0 applied with 18:1 was 40 and 45%, respectively. These values are considerably greater than were obtained when [^{14}C]16:0 was applied alone (Fig. 1).

The fact that [^{14}C]16:0 not incorporated during the first few hours following application in DGME remained largely unchanged for days suggested that these molecules were for some reason unavailable for ready usage. No enhancement of uptake could be gained by applying, after 30 min and again after 110 min, additional microdroplets of pure DGME to the exact spots where the radiotracer had been placed. Swabbing the surface of prelabeled leaves with absorbent paper wet with a mild detergent solution did not remove a significant amount of radioactivity. However, dipping a leaf prelabeled for 48 h with [^{14}C]16:0 into cold hexane for 45 s removed 53% of the total radioactivity associated with the leaf, and 96% of this was present as free FA. A second wash of the same leaf, this time with cold chloroform, removed another 21% of the total radioactivity, of which 92% was found to be free FA. A negligible amount of polar lipids or Chl was extracted from the leaf by these treatments.

Further tests with DGME revealed that 0.2 μl droplets of the solvent evaporated from glass or plastic surfaces in the growth chamber in approximately the same time period as required for the disappearance of droplets placed on the leaves. It seems probable that little if any of the DGME is actually absorbed through the spinach cuticle into the cells and that the [^{14}C]16:0 is simply deposited on the leaf surface, where a certain fraction finds its way into metabolically active compartments. Indeed, a leaf labeled by lightly rubbing its upper surface with a spatula upon which 1 μCi of [^{14}C]16:0 had been deposited by solvent evaporation had incorporated, by 7 d, 24% of the label applied. This degree of utilization is equivalent to that achieved through the use of DGME. The lipid distribution of radioactivity was in this case also similar to that achieved using DGME with, for example, 41% of the MGD radioactivity being accounted for by [^{14}C]16:3.

Once it appeared that DGME did little to enhance [^{14}C]16:0 uptake by spinach leaves, an improved solvent was sought. By dissolving [^{14}C]16:0 in liquid paraffin and applying it as a very thin film to the leaf, a much greater uptake was achieved. At 2, 4, and 24 h following application of 0.5 μCi [^{14}C]16:0 to 0.2 g leaves, 88, 94, and 97%, respectively, of the administered FA had been esterified into lipids. The pattern of incorporation into polar lipids was very similar to that found in the earlier experiments using DGME, with a steady increase in galactolipid radioactivity coinciding with a drop in PC labeling. Specifically, after 2 h, 4 h, 1 d, and 3 d of labeling, the incorporation figures, as percent of total dpm, were MGD: 3,3,6,8; DGD: 0.6,2,5,8; PC: 33,32,24,16. In MGD purified from leaves labeled for 3 d with [^{14}C]16:0 in liquid paraffin, 16:3 contained 61% of the total radioactivity, as determined by reverse phase TLC of FAME.

Incorporation of [^{14}C]Palmitate by Other Plant Species. A few experiments were conducted using another 16:3 plant, *Arabidopsis thaliana*, and an 18:3 plant, *Pisum sativum*. [^{14}C]16:0 applied to *Arabidopsis* leaves in DGME was poorly absorbed, with 22 and 31% being present in esterified form by 1 and 4 d, respectively. However, a larger uptake was achieved when [^{14}C]16:0 was applied in liquid paraffin. Radioactive FAME derived from MDG of the paraffin-labeled leaf were distributed among all the bands representing polyunsaturates on reverse phase TLC plates, with 15% of the total comigrating with reference 16:3.

Pisum leaves treated with [^{14}C]16:0 in DGME utilized the tracer poorly, as did the other two species, with only 24% being incorporated into ester linkage after 7 d. MGD and DGD con-

tained 1.8 and 1.9%, respectively, of the total extracted radioactivity at this time. Less than 0.5% of the MGD and DGD radioactivity was associated with the 16:3 FAME band on reverse phase TLC plates. Much of the radioactivity, especially in DGD, was localized in the 16:0 band, but there were indications of considerable labeling in C_{18} FA, *i.e.* 18:2 and 18:3, of MGD. Catalytic hydrogenation of the *Pisum* MGD and total *Pisum* phospholipids from leaves labeled more efficiently by applying [^{14}C]16:0 in liquid paraffin, followed by reverse phase TLC of the saturated FAME derived from them, showed that stearic acid present in the hydrogenated products accounted for approximately 60% of MGD FAME radioactivity and 20% of phospholipid radioactivity with palmitic acid making up the rest. We have not determined whether *Pisum* elongates [^{14}C]16:0 in its chloroplasts or in a cytoplasmic compartment perhaps involved in wax ester formation.

DISCUSSION

The data presented above indicate that exogenous ^{14}C-FA applied to spinach leaves are incorporated into all major glycerolipids. Although when applied in DGME only a relatively small percentage of the most informative tracer, [^{14}C]16:0, was utilized by the leaves, that material was incorporated quickly and mainly into lipids formed by microsomal enzymes. Thus, it served essentially as a pulse labeling of the cells. A much larger fraction of the same radioisotope applied in liquid paraffin was incorporated into lipids over the same time interval. With the continued passage of time, radioactivity was gradually transferred to eukaryotic galactolipids by well known pathways and to prokaryotic galactolipids by mechanisms which have not previously been recognized.

The use of exogenous ^{14}C-FA tracers to study intracellular FA fluxes in plants may be questioned since FA do not normally enter the cells in this way. By finding that a variety of application protocols all yielded similar patterns of incorporation (although the extent of incorporation varied) we have concluded that the tracers did enter natural metabolic pathways and that our results are not merely an artifact of the application method or quantity of FA absorbed. The key indicator of prokaryotic lipid synthesis, [^{14}C]16:3, was present when DGME or liquid paraffin were used as solvents and also when no solvent was used.

The findings described above run counter to the prevailing idea that acyl groups from extrachloroplastidal sources are unavailable for incorporation into prokaryotic lipids of higher plants. That concept is based upon three main lines of evidence. First, [^{14}C]16:0 was apparently not further metabolized, apart from some oxidation, following its incorporation into leaves of various plants (2, 15, 17). While this would seem to argue against 16:0 being able to enter the *sn*-2 position of prokaryotic lipids, the plant species chosen for the studies happened to be 18:3 plants, which we now recognize as lacking the enzymes for placing even *in situ* synthesized 16:0 into any of these lipids except PG.

Second, [^{14}C]18:1 and [^{14}C]18:2 applied to expanding leaf tissues, whereas rapidly incorporated into PC, were apparently inconsequentially incorporated into the prokaryotic lipid PG (16, 21, 24, 29). We also observed a strikingly lower incorporation into prokaryotic lipids of [^{14}C]18:1 as compared with [^{14}C]16:0, but we propose in the following discussion a plausible reason why these differences might exist. Third, *in vitro* studies with spinach chloroplasts (3, 24) have been interpreted to mean that FA-ACP is required as the immediate donor of acyl chains for prokaryotic lipid formation.

Our experiments, all performed using attached leaves, indicate an active incorporation of exogenous [^{14}C]16:0 into lipids formed by the prokaryotic biosynthetic pathway. We believe that the clear demonstration of FA esterification into these lipids was possible because (a) a plant species having an active prokaryotic pathway for lipid synthesis was utilized for the studies, and (b) [^{14}C]16:0 is a much more sensitive indicator of these fluxes than are C_{18} FA.

Two mechanisms for the entry of ^{14}C-FA into prokaryotic lipids can be envisioned. The first would involve entry of free ^{14}C-FA into the chloroplast, either directly after its uptake or from an extrachloroplastidal free FA pool in equilibrium with rapidly turning over phospholipid acyl chains. Considerable dilution of either [^{14}C]16:0 or [^{14}C]18:1 specific activity would result when *de novo* synthesized 16:0 and 18:1 mixed with the absorbed tracer, but the dilution of [^{14}C]18:1 would be much greater. It can be calculated from the known FA composition of spinach GL (4, 8) that the synthesis of eukaryotic GL molecular species requires 27 times more C_{18}FA than C_{16}FA. This greater flux of C_{18}FA through the cytoplasm and into the chloroplast as eukaryotic GL should carry with it the majority of [^{14}C]18:1 molecules entering the free FA pool in our experiments. But because C_{16}FA are discriminated against in eukaryotic GL synthesis, exogenously supplied [^{14}C]16:0 should remain longer in the free FA pool and would consequently be more often randomly drawn into the chloroplast.

A second possible mode of [^{14}C]16:0 entry into lipids having prokaryotic characteristics would involve its movement into chloroplasts via the usual pathway for eukaryotic GL formation, followed by its desaturation to 16:3 either before or after its placement in the *sn*-2 position of GL. This unorthodox pathway cannot at present be ruled out, although it is difficult to explain why [^{14}C]16:0 but not [^{14}C]18:1 should become incorporated into MGD molecular species of the prokaryotic type by this route.

Our findings represent the first *in vivo* study of [^{14}C]16:0 incorporation by higher plants containing prokaryotic glycolipids. While the data presented here leave open the question of how quantitatively significant the reverse flux of FA into prokaryotic lipids is, they clearly establish that a sizable flux can and does occur. Previous work with *Dunaliella* (18, 22) suggested that the physiological importance of particular FA movements from one cellular compartment to another may become much greater under certain types of environmental stress. It now seems credible that in higher plants, too, prokaryotic lipid production is not strictly dependent upon newly synthesized FA for its supply of acyl groups but may draw them from other sources within the cell when needed. The possible utility of this flexibility remains to be determined.

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