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Manipulating the Incorporation of [1-^{14}C]Acetate into Different Leaf Glycerolipids in Several Plant Species

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ABSTRACT

During short term labeling of expanding leaves of seven plant species with [1-^{14}C]acetate, 35 to 64% of the label incorporated into lipids was found in phosphatidylcholine and 5 to 24% in phosphatidylglycerol. In pumpkin, sunflower, broad bean, and maize, only 4 to 12% of the label was found in diacylgalactosylglycerol, but in tomato, parsley, and spinach, the proportion was 17 to 31%. The latter group was further distinguished by having diacylgalactosylglycerol containing C16:3.

The proportions of total incorporated [1-^{14}C]acetate entering the lipids could be manipulated in a predictable manner. Phosphatidylcholine labeling was depressed by treating intact leaves with glycerol or ethylene glycol monomethyl ether or by incubating leaf discs *in vitro*. An associated increase in phosphatidylglycerol labeling occurred within the first group of plants, whereas an increase in labeling of either diacylgalactosylglycerol, phosphatidylglycerol, or sulfolipid occurred within the second group. Treating intact leaves with glycerol or incubating leaf discs *in vitro* was shown to elevate cellular concentrations of *sn*-glycerol 3-phosphate.

These results have been interpreted in terms of the two-pathway hypothesis for glycerolipid biosynthesis, in which it is proposed that phosphatidylcholine is synthesized via a different pathway (eukaryotic) to that for synthesis of phosphatidylglycerol (prokaryotic). Both pathways may contribute toward the synthesis of diacylgalactosylglycerol, with the contribution of each being assessed from the proportion of hexadecatrienoic acid found in the particular plant.

Labeling leaves of broad bean with $^{14}CO_2$ (6, 29) or the leaves of sunflower, sorghum, or pumpkin with [1-^{14}C]acetate (15) initially gave rise to a rapid accumulation of radioactivity in PC[1] and PG. The label in PC was located predominantly in C18 unsaturated fatty acids, whereas the C16 fatty acids, palmitate, and 3-transhexadecaenoate as well as unsaturated C18 fatty acids were highly labeled in PG (15, 29). Analogous results have been reported for [1-^{14}C]acetate incorporation after longer labeling times in chervil (*Anthriscus*), a 16:3 plant (22), where radioactivity accumulated predominantly in C18 fatty acids of PC, but in both C16 and C18 fatty acids of DGG as well as PG. Whereas the proportions of radioactivity incorporated into the different leaf glycerolipids appear to be constant for a particular species under given growing conditions, there are indications that the labeling pattern may be amenable to manipulation. Thus, the PC:PG:DGG labeling ratio of 45:15:20 found in spinach leaves (17) was altered to 21:15:43 in leaf discs labeled *in vitro* (Willemot, C., Slack, C. R., Browse, J., and Roughan, P. G., in press) and the PC:PG labeling ratio of 5 to 6 in field-grown pumpkin was reduced to 1.5 to 2.2 in pot-grown plants (15). This may account for the large within-species variation in lipid labeling pattern reported by one laboratory (2, 4, 28).

Isolated spinach chloroplasts synthesize predominantly unesterified fatty acids from supplied [1-^{14}C]acetate (16), with diacylglycerol accounting for 20 to 25% of incorporated label. In the presence of either *sn*-G3P or Triton X-100, the proportion of [1-^{14}C]acetate entering diacylglycerol was increased and *sn*-G3P decreased the proportion of labeled oleate:palmitate synthesized (10, 16, 20). This ability of isolated chloroplasts to divert newly synthesized fatty acids into either unesterified fatty acids or into 1,2-diacylglycerol has led to the suggestion that two main routes of glycerolipid synthesis are possible. These pathways would give rise to lipids either poor or rich in C16 fatty acids (16, 17, 19) and the branchpoint would be located in the chloroplast.

The free fatty acids synthesized by isolated chloroplasts, which consisted of 75 to 80% oleic acid, were 66% converted to [^{14}C]acyl-CoAs in the presence of exogenous CoA and ATP (20) and have been considered to be the most likely precursors for PC synthesis *in vivo* (17, 19). This high oleic acid content of the free fatty acid fraction and its derivative acyl-CoA is in marked contrast with the fatty acid disposition of 1-oleoyl-2-palmitoyl or 1,2-dipalmitoyl found both in the precursor diacylglycerols synthesized *in vitro* (10, 17) and the DGG and PG derived from them *in vivo* (15, 17, 23, 29).

If there are indeed two separate pathways for glycerolipid synthesis, as is suggested by the fatty acid metabolism of isolated chloroplasts, it should be possible to promote selectively and predictably the flux of fatty acids synthesized from [1-^{14}C]acetate into different glycerolipids *in vivo*. This paper reports experiments which confirm this prediction.

MATERIALS AND METHODS

Plant Material. All analyses were performed on expanding leaves of plants. Spinach (*Spinacia oleracea*) was grown hydroponically (25); sunflower (*Helianthus annuus*), tomato (*Lycopersicum esculentum*), broad bean (*Vicia faba*), maize (*Zea mays*), and zucchini (*Cucurbita pepo*) were grown in the glasshouse; pumpkin (*Cucurbita pepo*), parsley (*Petroselinum hortense*), and in some cases sunflower and tomato were grown in the field.

Substrates and Chemicals. [1-^{14}C]Acetate (56.7 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, analytical reagent grade glycerol was from British Drug House Ltd., and EGMME was from Mallinckrodt.

Tissue Treatments and Substrate Incorporation. [1-^{14}C]Acetate (56.7 mCi/mmol) and other compounds were either supplied to attached leaves by application to the surface (details in the tables)

[1] Abbreviations: PC, phosphatidylcholine; PA, phosphatidic acid; PI, phosphatidylinositol; SL, sulfolipid; DDG, diacyldigalactosylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DGG, diacylgalactosylglycerol; *sn*-G3P, *sn*-glycerol 3-phosphate; TP, triose phosphate; EGMME, ethylene glycol monomethyl ether, ACP, acyl carrier protein.

or to detached leaves by petiolar uptake. Leaves which wilted during petiolar uptake of precursors were discarded, and glycerol (50 mM) administration was for 0.5 to 1.5 h prior to supply for 1 to 1.5 h of 2 to 10 μCi of [1-^{14}C]acetate in a volume of 25 to 500 μl water or 50 mM glycerol. For tissue labeling *in vitro*, 50 discs (diameter, 2 mm) cut at random from leaves were vacuum-infiltrated and incubated at 25°C in 0.5 ml 10 mM Mes/NaOH, pH 5.5, containing 0.33 M sorbitol and 2 μCi [1-^{14}C]acetate (56.7 mCi/mmol) as specified, in a shaking water bath with illumination.

Lipid Analysis. Lipids were extracted by the technique of Folch *et al.* (3), care being taken to minimize phospholipase D activity, either by steam-killing the material prior to homogenization, or by rapid extraction using a Polytron homogenizer (Kinematica, Sweden) (21). Glycerolipids were separated from total lipid extracts (equivalent of 50–100 mg fresh weight of leaf) by two-dimensional TLC (21), detected by staining lightly with iodine vapor and individual lipid-containing regions scraped into 8 ml of 3.3% (w/v) *p*-terphenyl in xylene:Triton X-100:water (2:1:0.1 by volume) for scintillation counting. Neutral lipids which contributed 10% (spinach) to 20% (broad bean) of total lipid radioactivity (intact leaves) were omitted from the analysis. Hexadecatrienoic acid (C16:3) content of DGG isolated from total lipids by TLC with the solvent system chloroform:methanol:acetone (80:15:5, v/v) was determined by GLC following methylation with sodium methoxide. Radioactively labeled molecular species of similarly isolated DGG were separated by TLC on activated silica gel containing 5% $AgNO_3$ with the solvent system chloroform:methanol:acetone (80:15:5, v/v) and detected by radioautography. Radioactivity in individual fatty acid methyl esters from total lipid extracts was determined following transmethylation with sodium methoxide and separation of fatty acid methyl esters by argentation TLC. *sn*-G3P and TP levels were assayed spectrophotometrically (9) in extracts (27) prepared from 18 discs (diameter, 15 mm) cut rapidly from attached illuminated leaves and immediately frozen in liquid air. Chl was determined (1) in an 80% acetone extract from six discs cut from the same leaves.

RESULTS

Lipid Labeling Patterns in Two Plant Groups. There were differences between species in the relative amounts of label incorporated into PC, DGG, and SL from [1-^{14}C]acetate supplied for short periods to the leaf surface, and the plants appeared to fall into two distinct groups (Table I). Low DGG and SL labeling and high PC labeling was found in pumpkin, sunflower, and broad bean, whereas higher labeling of DGG and SL and lower PC labeling was found in tomato, parsley, and spinach. This difference was associated with the presence of a C16:3 acyl group in the DGG of the latter group of plants. Tomato, parsley, and spinach contained respectively 56, 76, and 50% of the DGG as the 16:3/18:3 molecular species, whereas in broad bean (4), pumpkin, and sunflower, as well as zucchini and maize, this species of DGG was not detected (see "Materials and Methods"). Representatives of the two groups of plants also showed distinct differences in the molecular species of DGG seen after short term labeling (Fig. 1). Molecular species of DGG found in spinach included C16:0/18:1, C16:0/18:2, C16:0/18:3, C18:2/18:3, C16:2/18:3, C16:3/18:3, C18:2/18:2, and C18:3/18:3, whereas C18:2/18:2 and C18:3/18:3 were found to be labeled in zucchini.

Effect of Incubation *In Vitro*. Feeding [1-^{14}C]acetate to leaf discs rather than to intact leaves resulted in a reduction of the proportion of label incorporated into PC in all cases (Table I). In the non-16:3 plants, the discs contained an increased proportion of the label in PG. In these species, this lipid is the only glycerolipid which contains C16 fatty acids esterified at position 2. In the 16:3 species, on the other hand, where DGG and SL as well as PG contain a C16 fatty acid at position 2, one or all of these three lipids contained an elevated amount of label in discs compared with the intact leaf.

Tissue *sn*-G3P Content. The *sn*-G3P content within spinach leaves more than doubled following incubation *in vitro* of small leaf discs or treatment with 50 mM glycerol, whereas treatment with EGMME had no effect on *sn*-G3P levels (Table II). The TP concentration in intact leaves, which was comparable to that

Table I. *Radioactivity in Lipid Classes after Labeling In Vivo of Whole Leaves Compared with Labeling In Vitro of Discs*

For labeling *in vivo*, 1 to 10 μCi [1-^{14}C]acetate was applied in 10 to 500 μl water to half or the whole of the surface of the leaf, either with a micropipette (small volumes) or a brush (large volumes). Discs for analysis of labeling pattern *in vitro* were taken by random sampling of leaves of a similar growth stage to controls, or the unlabeled half of larger leaves. All plants except for broad bean and spinach were field grown (see "Materials and Methods").

Plant	Time	Labeling	Distribution							
			PA	PI	PC	SL	DDG	PG	PE	DGG
	h						%			
Pumpkin	0.5	Leaf surface	2.2	7.4	63.1	0.7	3.9	8.6	9.9	4.2
	1.0	Discs	1.9	7.1	52.8	1.0	1.0	15.3	10.5	10.4
Sunflower	0.5	Leaf surface	3.5	1.8	63.6	1.3	1.8	12.6	3.8	11.6
	2.0	Discs	4.6	3.5	42.0	0.7	8.5	26.7	6.9	7.6
Broad bean	0.5	Leaf surface	1.4	3.8	61.5	0.5	1.0	18.7	4.3	9.6
	1.0	Discs	4.9	6.9	44.9	0.7	4.2	30.7	4.4	3.4
Tomato	1.0	Leaf surface	2.3	4.3	39.2	5.5	2.1	13.9	4.8	27.9
	1.0	Discs	4.2	4.4	20.2	9.3	4.9	18.4	4.7	33.9
Parsley	1.2	Leaf surface	2.0	4.2	35.7	5.0	4.8	11.0	0.2	35.5
	1.0	Discs	2.0	1.4	28.6	2.8	3.8	24.9	4.3	32.4
Spinach	0.5	Leaf surface	0.9	3.1	46.0	3.1	2.6	12.6	4.2	27.4
	1.0	Discs	1.1	2.8	21.5	7.8	3.2	13.3	3.9	46.7

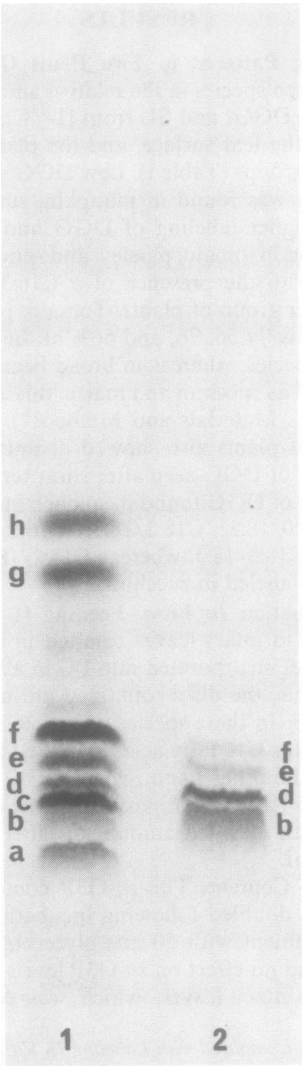

FIG. 1. The different molecular species of DGG synthesized by a 16:3 and a non-16:3 plant. Expanding leaves of spinach and zucchini were labeled *in vivo* with 10 μCi [1-^{14}C]acetate for 2 h. Radioautogram is of DGG species separated by argentation chromatography (see "Materials and Methods"). 1, Spinach; 2, zucchini. Tentative identification of DGG species (23) are (a) C18:3/16:3, (b) C18:3/18:3, (c) C18:3/16:2, (d) C18:3/18:2, (e) C18:2/18:2, (f) C18:3/16:0r, (g) C18:2/16:0, and (h) C18:1/16:0.

previously found by Stitt *et al.* (27), fell considerably on incubation of discs *in vitro*.

Effect of Glycerol and EGMME Application on the Distribution of Radioactivity among Different Glycerolipids. In the isolated spinach chloroplasts, *sn*-G3P appears to act as a fatty acyl chain terminator, by diverting palmitoyl-ACP directly into glycerolipid synthesis rather than chain elongation. With increasing concentrations of *sn*-G3P, the proportion of oleate to palmitate synthesized *de novo* decreases, the palmitate being incorporated predominantly into diacylglycerol (10, 16, 20). Because of this and the observed increase in spinach discs of the steady-state level of *sn*-G3P (Table II), together with glycerolipids derived from chloroplast diacylglycerol (Table I), we attempted to increase the level of tissue *sn*-G3P in intact leaves by supplying glycerol to the leaf. Application of 50 mM glycerol to the surface produced an *sn*-G3P level comparable to that of discs and about 2.5 times higher than that of controls (Table II) and in addition markedly altered the distribution of radioactivity among the different lipids in a pattern analogous to that produced by incubating leaf discs *in vitro* (Table III). There was no effect on total incorporation. Decreased labeling

Table II. *Content of sn-Glycerol 3-Phosphate and Triose Phosphates in Spinach*

Extracts from control and pretreated leaves were assayed for *sn*-G3P or TP as outlined in "Materials and Methods." Small discs (100 of 2-mm diameter) were cut from the half-leaves remaining from control determinations (6 leaves/experiment) and preincubated for 1 h as outlined in "Materials and Methods" for labeling *in vitro* (in the absence of [1-^{14}C] acetate). Other leaves were wet to run-off with 50 mM glycerol or spread with 100 μl EGMME/leaf (3 leaves/treatment) 1 h before harvest.

Treatment	G3P	TP
	nmol/mg Chl	
Control	11	53
Discs	27	11
Glycerol	27	56
EGMME	13	52

in PC was compensated for by increased labeling in PG (sunflower and broad bean) or increased labeling in DGG (spinach). There was a difference in the sunflower PC/PG labeling ratio between field-grown (Table I) and pot-grown plants (Table III).

The effect of glycerol on distribution of radioactivity into the different glycerolipids was further investigated by petiolar uptake studies with intact excised leaves of four species. In maize, sunflower, and parsley, glycerol depressed incorporation of [1-^{14}C] acetate into PC but increased the labeling of PG, whereas in tomato, incorporation into DGG increased from 28 to 46% (data not shown). The observed changes in glycerolipid labeling in sunflower were associated with an increase in proportion of C16 in total lipids, with the C18 total: C16:0 labeling ratio changing from 4.6 in controls to 1.7 in leaves fed glycerol via the petiole. In contrast to glycerol, sucrose had no effect on labeling pattern (data not shown). [1-^{14}C]Acetate application in 75% (v/v) EGMME led to similar changes to those caused by glycerol, also without appreciably affecting total incorporation (Table III).

DISCUSSION

Labeling Patterns and Two-Pathway Hypothesis. The present results show that [1-^{14}C]acetate incorporation into plant lipids *in vivo* can be manipulated in a manner consistent with the two-pathway hypothesis that we have recently proposed (17, 19). According to this scheme, two alternative pathways for glycerolipid synthesis are possible. One pathway ('eukaryotic') involves microsomal PC as an intermediate in the synthesis of DGG containing predominantly linolenate (14, 25, 29) and with the 2-position occupied exclusively by this fatty acid (24). The other pathway ('prokaryotic') leads to the formation within the chloroplast of 1-linolenyl-2-hexadecatrienoyl DGG (18, 22) as well as PG and SL containing C16 fatty acids at the 2-position. This is the only pathway operating in blue-green algae (12, 30) and the capacity to synthesize DGG by this route is retained by relatively few angiosperm families (7).

The difference in biosynthetic pathways for DGG synthesis within the two types of plants is illustrated by the different molecular species of this galactolipid synthesized initially from [1-^{14}C]acetate (present results with Refs. 23 and 26). Several more saturated species of DGG which were relatively heavily labeled in spinach were virtually absent in zucchini and maize.

In all the plants examined in the present study which lacked C16:3 and hence had only the eukaryotic pathway for DGG synthesis, conditions which depressed incorporation of [1-^{14}C]acetate into the typically eukaryotic lipid PC (incubation *in vitro* of discs, glycerol, or EGMME application) resulted in a concomitant elevation of labeling of PG (typically prokaryotic). In all instances but one (pumpkin), the labeling of DGG, which according to our hypothesis is synthesized via PC in these plants, also fell. This

Table III. *The Effect of Glycerol and EGMME on Distribution of Radioactivity among Lipid Classes after Surface Labeling of Leaves*

Leaves of pot-grown sunflower and broad bean were pretreated with 50 mM glycerol by wetting to run-off, while application to spinach was in the form of microdroplets (150 μl total volume). One to 10 μCi [1-^{14}C]acetate was applied 45 to 60 min later in 10 to 250 μl water or in 20 μl 75% (v/v) EGMME. Lipids were extracted 0.5 h after application of radioactivity.

Plant	Treatment	Distribution							
		PA	PI	PC	SL	DDG	PG	PE	DGG
						%			
Sunflower	Control	1.7	4.7	51.6	5.9	1.1	23.5	4.1	7.4
	Glycerol	1.8	3.7	33.0	7.1	2.8	42.2	2.6	6.8
Broad bean	Control	1.4	3.8	61.5	0.5	1.0	18.7	4.3	9.6
	Glycerol	6.3	4.2	41.2	0.5	0.5	38.2	5.1	4.2
	EGMME	0.7	2.0	49.7	0.7	1.0	33.0	5.0	8.1
Spinach	Control	0.9	3.1	46.0	3.1	2.6	12.6	4.2	27.4
	Glycerol	0.4	2.9	23.3	6.5	3.7	6.8	2.8	42.0
	EGMME	0.3	2.3	22.6	5.2	2.9	4.5	2.6	46.6

indicates that the fall in PC labeling was not due to an increased rate of transfer of radioactivity to DGG from precursor PC. The doubling in palmitate content of the total radioactively labeled lipids synthesized in sunflower leaves after glycerol application reinforces the concept that the balance of the two pathways was being altered by the glycerol, as lipids synthesized by the prokaryotic pathway via chloroplast diacylglycerol (17, 19) contain a higher proportion of C16 fatty acids (15, 22, 29). The labeling of DGG was higher and the changes in labeling pattern were more complex in the case of the plants which retain the capability to synthesize DGG via the prokaryotic pathway as well as the eukaryotic pathway (16:3 plants). However, all glycerolipids which showed elevated labeling when incorporation into PC was depressed (variously one or more of DGG, PG, and SL) are synthesized via the prokaryotic pathway.

Mode of Action of Perturbants. Glycerol application consistently caused a proportionate decrease in incorporation of [1-^{14}C] acetate into PC and an increase in labeling of some or all of the lipids PG, DGG, or SL according to plant type. It also caused a concomitant increase in the radioactivity in palmitate relative to oleate in total glycerolipids of sunflower. An increase in diacylglycerol synthesis and in the palmitate/oleate ratio was previously noted on addition of *sn*-G3P to isolated chloroplasts (10, 16, 20). These effects, correlated with the present observed action of supplied glycerol in raising the tissue *sn*-G3P in spinach, are consistent with the suggestion that the initial reaction of palmitoyl-ACP is a direct transfer of the palmitoyl moiety to *sn*-G3P (17, 19). According to the model proposed for chloroplast lipid biosynthesis, the palmitoyl-*sn*-glycerol-3-P thus formed would then be further acylated from acyl-ACP to form phosphatidic acid, dephosphorylated to diacylglycerol (8), which would be the precursor for DGG (16:3 plants only) (10, 17, 19), PG, and SL. Hence, the present effects of glycerol in altering the glycerolipid-labeling pattern can be accounted for in terms of its observed effect in raising tissue *sn*-G3P levels. The analogous effect of incubation *in vitro* on incorporation in leaf discs is predicted to be due to the observed increase in concentration of cellular *sn*-G3P which in turn may have resulted from a build-up of photosynthate (*cf.* Ref. 15; and the reduction in PC/PG ratio in pot-bound sunflower compared to field-grown plants observed in the present results).

The surfactant EGMME failed to have any effect on tissue *sn*-G3P concentration and is therefore likely to have a different mode of action from glycerol. Spreading agents have recently been used in studies of lipid synthesis to aid precursor uptake (5, 11, 13). Inasmuch as EGMME causes such a marked change in lipid metabolism, we would caution against its uncritical use.

We conclude that the present results using intact leaf tissue provide support for an integrated scheme for glycerolipid biosynthesis involving interplay of two pathways, one centered in the cytoplasm and the other in the chloroplasts, which was originally proposed on the basis of studies with isolated chloroplasts (17, 19).

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