The Kinetics of Incorporation *in vivo* of [^{14}C]Acetate and [^{14}C]Carbon Dioxide into the Fatty Acids of Glycerolipids in Developing Leaves

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(Received 1 April 1975)

1. The patterns of incorporation of ^{14}C into glycerolipid fatty acids of developing maize leaf lamina from supplied [$1\text{-}^{14}\text{C}$]acetate and from $^{14}\text{CO}_2$ during steady-state photosynthesis were similar. Oleate of phosphatidylcholine and palmitate of phosphatidylglycerol attained linear rates of labelling more rapidly than did other fatty acids, particularly the linoleate and linolenate of monogalactosyl diacylglycerol. 2. After the transfer of lamina from labelled to unlabelled acetate, there was a decrease in labelled oleate and linoleate of phosphatidylcholine and a concomitant increase in the amount of radioactivity in the linoleate and linolenate of monogalactosyl diacylglycerol. 3. The rapidly labelled phospholipids, phosphatidylcholine and phosphatidylglycerol, were shown by differential and sucrose-density-gradient centrifugation to be associated with different organelles, the former being mainly in a low-density membrane fraction, probably microsomal, and the latter mainly in chloroplasts. 4. During a 48 h period after supplying spinach leaves with [^{14}C]acetate, radioactivity was lost from the oleate of phosphatidylcholine present in fractions sedimented at 12000 g and 105000 g, and accumulated in the linolenate of monogalactosyl diacylglycerol of the chloroplast. 5. It is proposed that the phosphatidylcholine of some non-plastid membranes is intimately involved in the process of oleate desaturation and that this lipid serves as a donor of unsaturated C_{18} fatty acids to other lipids, principally monogalactosyl diacylglycerol, of the chloroplasts.

Linolenate, the major fatty acid of photosynthetic tissues, constitutes approx. 90% of the fatty acids of the galactolipids (O'Brien & Benson, 1964) that are mainly localized in the chloroplast lamellae (James & Nichols, 1966). However, neither the mechanism nor the intracellular site of linolenate biosynthesis in photosynthetic tissues has been established and many of the published results on fatty acid desaturation in leaves and algae are in conflict. The early time-course studies by James (1963) demonstrated that [^{14}C]acetate was readily incorporated into leaf fatty acids, and suggested that linolenate was formed from oleate, via linoleate, by sequential desaturation. This view has been supported by the observation that specifically labelled oleate, when fed to leaves and algae, is converted first into linoleate and then into linolenate without apparent degradation of the carbon chain (Harris & James, 1965; Klenk, 1972; Tremolieres & Mazliak, 1974). Stumpf and co-workers, however, interpret the results of labelling experiments with leaf tissue (Kannangara *et al.*, 1973) and disrupted chloroplasts (Jacobson *et al.*, 1973) as indicating that linoleate is formed by the desaturation of oleate, but that linolenate is produced by chain elongation from a dodecatrienoic acid.

From the rates of change of the specific radioactivities of particular fatty acids in the individual lipids of cells of *Chlorella vulgaris*, pulse-fed with [^{14}C]acetate, it was suggested that certain lipids,

particularly monogalactosyl diacylglycerol and phosphatidylcholine, could be involved in the further desaturation of oleate (Nichols *et al.*, 1967). However, Nichols and co-workers (Nichols, 1968; Appleby *et al.*, 1971) interpreted the results of the later studies as indicating that labelled linoleate and linolenate in the monogalactosyl diacylglycerol of *Chlorella vulgaris* cells was derived only from labelled oleate previously incorporated into this lipid. This view is not in accord either with the finding that oleate is incorporated preferentially into the phosphatidylcholine of *Chlorella vulgaris* (Gurr *et al.*, 1969; Gurr & Brawn, 1970) or with the observation that the radioactivity initially incorporated into oleate of phosphatidylcholine of pumpkin leaves, pulse-fed with [^{14}C]acetate, subsequently accumulate in linoleate and linolenate of monogalactosyl diacylglycerol (Roughan, 1970, 1975).

The labelling studies *in vivo* described below support the view that phosphatidylcholine is involved in oleate desaturation and strengthens the proposal (Roughan, 1970) that non-chloroplast phosphatidylcholine may donate polyunsaturated fatty acids to monogalactosyl diacylglycerol of the chloroplasts.

Materials

Seedlings of maize (*Zea mays* cultivar Wisconsin 436) were grown in trays of pumice-peat in a heated

naturally lit glasshouse, and watered daily with nutrient solution. Leaves used in each experiment were from plants selected for uniformity on the basis of height and the extent of emergence of the youngest visible leaf when the fifth leaf was fully emerged. Strips of mature lamina (12 cm long, 1 cm wide) were cut under degassed water from each side of the midrib of the fifth leaves. Immature lamina was cut as above from partially expanded sixth leaves from the point of emergence to 12 cm above this point. Spinach plants (*Spinacea oleracea* cultivar King of Denmark) were grown in aerated nutrient solution (Hoagland & Arnon, 1938) in a controlled-environment cabinet at a temperature of 21°C, a light intensity of 140 W·m⁻² (400–700 nm) and a photoperiod of 10 h.

Pea seedlings (*Pisum sativum* cultivar Victory) were grown as described for maize and harvested 15 days after planting. Seedlings were selected in which the expanding leaflets were approximately one-half fully expanded, and their stems cut under degassed water below these leaflets.

A solution of sodium methoxide was prepared by dissolving 0.6 g of sodium in 50 ml of anhydrous methanol. Diazomethane was prepared by the method of Schlenk & Gellerman (1960). Sodium [1-¹⁴C]acetate and Na₂¹⁴CO₃ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Labelling of maize leaf lamina and pea shoots

Maize lamina strips or pea shoots were placed in a chamber (volume 3 litres) similar to that described by Hatch & Slack (1966), with their bases immersed in degassed water. Before supplying radioactive acetate the chamber was illuminated through a 3 cm-thick water screen by a 1 kW Philips halogen floodlight lamp at an intensity at the lamina surface of 130 W·m⁻². Air, saturated with water vapour at 28°C, was pumped through the chamber at 10 litres/min. Under these conditions the temperature at the leaf surface, monitored by a fine thermocouple, varied between 31° and 33°C. After 30 min the water was removed and replaced by 4.5 ml of 0.3 mM-sodium [1-¹⁴C]acetate (60 mCi/mmol). For measurements of the rate of labelling of lipids the individual leaves were removed at intervals; for cell-fractionation experiments, the leaves were removed and placed on ice. Alternatively, after 30 min the air supply was disconnected and the chamber attached to a reservoir (volume 10 litres) in which ¹⁴CO₂ had previously been generated from Na₂¹⁴CO₃. Air was pumped from the reservoir, through the chamber and back to the reservoir at 5 litres/min by using a gas-tight pump and thick-walled nylon tubing to minimize CO₂ loss. The initial ¹⁴CO₂ concentration was 0.2% (v/v) and the specific radioactivity 0.9 mCi/mol. Soft neoprene

seals along the abutting surfaces of the two halves of the chamber's lid, through which the maize lamina strips protruded, provided an adequate gas seal while allowing individual strips to be withdrawn at intervals.

Labelling of spinach leaves

At 30 min after the start of a photoperiod 80 µl of 0.5 mM-sodium [1-¹⁴C]acetate (60 mCi/mmol) in 80% ethanol was applied as 3 µl droplets to the surfaces of each of 12 expanding spinach leaves. These droplets evaporated in about 10 min. After 30 min the leaves were wiped several times with moistened tissue paper to remove unabsorbed acetate.

Leaf fractionation

Spinach leaves, harvested 1.5 h after the beginning of a photoperiod when little starch was present in chloroplasts, were placed on filter paper in ice-cold water and illuminated by a Philips 400 W HPL lamp at 50 W·m⁻². After 10 min the major veins were removed, the lamina diced and 10 g wet wt. was homogenized in 50 ml of partially frozen 50 mM-Mes*-NaOH buffer, pH 6.5, containing 330 mM-sorbitol, 5 mM-MgCl₂, 1 mM-MnCl₂, 2 mM-EDTA, 50 mM-KCl and 5 mM-sodium ascorbate with a VirTis 45 homogenizer (The VirTis Co., New York, N.Y., U.S.A.) at half-maximum speed for 2 s. The homogenate was filtered through three layers of Miracloth and the filtrate centrifuged successively at 2000 g for 15 s, 12000 g for 10 min and 105000 g for 1 h. Each pellet was resuspended in 1 ml of 50 mM-Hepes-NaOH buffer, pH 7.9, containing 330 mM-sorbitol, 1 mM-MgCl₂, 1 mM-MnCl₂, 2 mM-EDTA and 1 mM-dithiothreitol, and 0.05 ml samples were taken for chlorophyll determinations by the method of Arnon (1949).

Maize leaf laminae were fractionated as above except that the tissue was homogenized for 18 s to obtain adequate mesophyll cell breakage and chloroplasts were pelleted for 2 min at 2000 g.

Pea leaflets (1 g wet wt.) were chopped into 3 mm strips and homogenized in 10 ml of 100 mM-Hepes-KOH buffer, pH 7.5, containing 2 mM-MgCl₂, 2 mM-EDTA, 10 mM-KCl, 5 mM-dithiothreitol and 0.33 M-sorbitol in a Polytron PT 20 homogenizer (Kinematica, Lucerne, Switzerland) at half-maximum speed for 3 s. The homogenate was filtered through three layers of Miracloth and the filtrate centrifuged at 2000 g for 20 s. The pellet was treated essentially as described by Mackender & Leech (1974) to partially separate intact chloroplasts from broken plastids and mitochondria. The pellet was resuspended in 5 ml of the homogenization buffer and layered above 5 ml of the same buffer, in which sorbitol was replaced by

* Abbreviations: Mes buffer, 2-(*N*-morpholino)ethanesulphonic acid; Hepes buffer, 2-(*N*-2-hydroxyethyl-piperazin-*N'*-yl)ethanesulphonic acid.

0.4M-sucrose, and centrifuged at 500 g for 12 min. The lipids in the sorbitol and sucrose layers and the pellet were analysed separately. Portions (3ml) of the 2000 g supernatant were layered above a sucrose density gradient consisting of 16ml of a linear gradient from 32 to 54% (w/w) sucrose below 3 ml of 20% (w/w) sucrose and centrifuged in an MSE rotor (no. 59590) at 53000 g for 4 h at 2°C. The sucrose solutions also contained 50mM-Hepes-KOH buffer, pH 7.5, 1mM-MgCl₂ and 3mM-EDTA. The gradient was pumped through a flow cell (Uvicord II, LKB, Bromma, Sweden) and the transmission at 280nm recorded. Fractions (0.8ml) of the gradient were collected and the sucrose concentration of each was measured with a refractometer.

All the above procedures were carried out at between 0° and 4°C.

O₂ evolution by chloroplasts

The rate of O₂ evolution by samples of spinach chloroplast suspensions (containing approx. 100 µg of chlorophyll) was measured polarographically, essentially as described by Delieu & Walker (1972), in 2ml of the suspension medium used for spinach chloroplasts but without dithiothreitol and supplemented with 10mM-NaHCO₃.

Lipid and fatty acid analysis

Laminae were weighed, and then steamed before extraction as previously described (Roughan & Boardman, 1972). Organelle suspensions were extracted directly with 5 vol. of chloroform-methanol (2:1, by vol.). The chloroform layer was dried at 40°C, redissolved in chloroform, dried under O₂-free N₂, dissolved finally in chloroform containing 0.005% butylated hydroxytoluene, added as an antioxidant (Hitchcock & Nichols, 1971), and stored under N₂ at 4°C. Samples were counted for radioactivity in a gas-flow detector at a counting efficiency of 20%. Glycerolipids were separated by column chromatography (Rouser *et al.*, 1967) followed by t.l.c. on silica gel G in chloroform-methanol-acetic acid-water (85:15:10:3, by vol.). In some experiments lipid extracts were chromatographed directly on silica gel G thin-layer plates in chloroform-methanol-15M-NH₃ (65:25:2, by vol.) to separate monogalactosyl diacylglycerol, phosphatidylcholine and phosphatidylglycerol; other glycerolipids chromatographed together. Lipids were stained with I₂ vapour, and silica-gel zones containing individual lipids were transferred to scintillation vials each containing 10ml of scintillation fluid consisting of 0.5% *p*-terphenyl in xylene-Triton X-114-water (6:2:1, by vol.) and counted for radioactivity at an efficiency of 40%. Water was added to the scintillation fluid to deactivate the silica gel and facilitate the release of lipids into the organic phase as described previously (Roughan, 1970). Fatty acid methyl esters

were prepared from glycerolipids, separated on thin-layer chromatograms, lightly stained with I₂ vapour then destained in a stream of O₂-free N₂, either by the method of Roughan & Boardman (1972) or by incubating glycerolipids, plus silica gel, with 3ml of 0.5M-sodium methoxide in methanol for 15 min at room temperature (20°C), adding an equal volume of water to the reaction mix and extracting the methyl esters into light petroleum (b.p. range 40–60°C). The recovery of methyl esters from the two methods was essentially identical. Methyl esters were separated by g.l.c. at 180°C on columns (200cm×0.4cm) of Chromasorb W coated with 17% ethylene glycol succinate. Columns were fitted with effluent stream splitters so that 20% entered the flame-ionization detector and the remainder was diverted to a nozzle on to which glass tubes (75cm long, 0.4cm internal diameter), moistened internally with xylene, could be attached to collect individual esters. These were washed with scintillation fluid (0.5% *p*-terphenyl in xylene) into vials and counted for radioactivity at an efficiency of 55%. All data presented in the Results section were standardized to a 20% counting efficiency.

To determine the distribution of label within the oleate of phosphatidylcholine and linolenate of monogalactosyl diacylglycerol, these lipids were purified by preparative t.l.c. and their fatty acids methylated by using sodium methoxide as described above. The labelled methyl esters together with 25 µg of each of authentic methyl oleate, methyl linoleate and methyl linolenate were separated by 'argention' t.l.c. (Morris, 1966). The required methyl esters were eluted with ether, dried under N₂ and hydrogenated as described by Appelqvist (1972). Complete hydrogenation of the methyl esters of oleate and linolenate to methyl stearate was confirmed by g.l.c., and samples of the methyl stearate were saponified and subjected to α -oxidation, and the products were again methylated as described by Harris *et al.* (1965). The methyl esters were separated by g.l.c. as described above but at 150°C. Distribution of radioactivity between polar and acyl moieties of glycerolipids separated by t.l.c. was determined as described by Roughan (1970).

Results

Incorporation of [1-¹⁴C]acetate into maize leaf lipids

During time-course labelling experiments there was an increasing rate of entry of [¹⁴C]acetate into the glycerolipids of mature maize leaves (Fig. 1). Phosphatidylcholine, phosphatidylglycerol and monogalactosyl diacylglycerol contained most of the incorporated radioactivity and, in contrast with other lipids, phosphatidylcholine attained a linear rate of labelling after only a brief lag period. Essentially all the label in glycerolipids was isolated in the saponi-

Fig. 1. Time-course of incorporation of $[1-^{14}\text{C}]$ acetate into lipids of mature maize lamina

Strips of lamina from mature maize leaf were supplied with $[1-^{14}\text{C}]$ acetate as described in the Methods section. One strip was removed at each of the times shown after the labelled acetate had been supplied, and analysed. ●, Total lipids; ○, phosphatidylcholine; ▲, phosphatidylglycerol; △, monogalactosyl diacylglycerol.

Fig. 2. Time-course of incorporation of $[1-^{14}\text{C}]$ acetate into lipids of immature maize lamina

Lamina strips from the emerging sixth leaf of maize seedlings were supplied with $[1-^{14}\text{C}]$ acetate as described in the Methods section and sampled as in Fig. 1. ●, Total lipids; ○, phosphatidylcholine; ▲, phosphatidylglycerol; △, monogalactosyl diacylglycerol; ■, digalactosyl diacylglycerol + sulpholipid + phosphatidylethanolamine.

fiable fraction, and, during the first hour of labelling, oleate was the most highly labelled fatty acid of phosphatidylcholine and monogalactosyl diacylglycerol. The oleate of phosphatidylcholine was labelled at a linear rate after only a brief lag, which

contrasted with the accelerating rate of labelling of other fatty acids in this and other lipids. Little label was detected in linolenate.

The mature lamina used in the above experiment contained fully differentiated chloroplasts in which

Fig. 3. Time-course of labelling of the fatty acids of certain glycerolipids in immature maize lamina

The fatty acids of phosphatidylcholine (a), monogalactosyl diacylglycerol (b) and phosphatidylglycerol (c) labelled in the experiment described in Fig. 2 were analysed. ▲, Palmitate; ○, oleate; ●, linoleate; △, linolenate.

presumably the accumulation of galactolipids had ceased. Studies of plastid differentiation during leaf emergence in maize (Leech *et al.*, 1973) and other C_4 grasses (Slack *et al.*, 1974) have shown that chloroplast expansion and the accumulation of chloroplast lipids continue subsequent to the emergence of the leaf lamina from the sheathing leaf tissue. Consequently, to examine the pattern of incorporation of [^{14}C]acetate into glycerolipids during a period of net synthesis of galactolipids, we used recently emerged leaf lamina. The total amount of [^{14}C]acetate incorporated by this immature lamina (Fig. 2) was similar to that incorporated by mature lamina during a 2.5 h period, but, in the immature lamina, phosphatidylcholine, which again attained a linear rate of labelling before other lipids, and monogalactosyl

diacylglycerol contained more label relative to phosphatidylglycerol than in the mature tissue. The changes with time in the amount of radioactivity in the major labelled fatty acids of phosphatidylcholine, phosphatidylglycerol and monogalactosyl diacylglycerol (Fig. 3) showed some interesting differences compared with that for mature lamina. In phosphatidylcholine, oleate was initially the most heavily labelled fatty acid, but, in contrast with the mature tissue, the linoleate attained a rate of labelling similar to that of oleate 20 min after [^{14}C]acetate had been supplied, whereas the linolenate of phosphatidylcholine accumulated little radioactivity (Fig. 3a). Both the linoleate and linolenate of monogalactosyl diacylglycerol became labelled, after a lag of about 30 min, at a rate only slightly less than that for the

Table 1. *Changes in the distribution of radioactivity among the glycerolipids of immature maize leaf fed with [^{14}C]acetate then transferred to [^{12}C]acetate*

Ten lamina strips from the emerging sixth leaf were supplied with [^{14}C]acetate for 20 min as described in the Methods section, then transferred to 0.3 mM- ^{12}C]acetate. Two strips were bulked for lipid analysis at the time of transfer and at intervals during the subsequent 140 min.

Lipid	$10^{-5} \times$ Radioactivity in glycerolipids after feeding with [^{14}C]acetate and after transfer to unlabelled acetate (c.p.m./g wet weight)				
	[^{14}C]Acetate 20 min	[^{12}C]Acetate			
		30 min	50 min	105 min	140 min
Phosphatidylcholine	3.32	4.44	3.28	2.74	2.72
Phosphatidylglycerol	1.46	2.10	1.98	2.12	1.78
Monogalactosyl diacylglycerol	1.08	2.04	1.72	2.72	3.28
Digalactosyl diacylglycerol+phosphatidyl- ethanolamine+sulpholipid	0.46	0.76	0.88	1.32	1.58

Table 2. *Specific radioactivity of the degradation products of the oleate of phosphatidylcholine and linolenate of monogalactosyl diacylglycerol from maize lamina fed with [^{14}C]acetate then transferred to [^{12}C]acetate*

Phosphatidylcholine and monogalactosyl diacylglycerol were purified from lipid extracts, described in Table 1 and Fig. 3, of lamina supplied with [^{14}C]acetate for 20 min and of lamina supplied with [^{14}C]acetate for 20 min then transferred to [^{12}C]acetate for 140 min. The oleate of the former lipid and linolenate of the latter lipid were purified, hydrogenated, degraded by α -oxidation, and their degradation products separated as described in the Methods section. The specific radioactivity is c.p.m./relative mass estimated from gas-chromatograph trace, peak height $\times$ retention time.

Degradation product	Specific radioactivities of the degradation products of oleate and linoleate			
	[^{14}C]Acetate for 20 min		[^{12}C]Acetate for 140 min	
	Phosphatidylcholine oleate	Monogalactosyl diacylglycerol linolenate	Phosphatidylcholine oleate	Monogalactosyl diacylglycerol linolenate
$C_{14:0}$	20.7	0.4	2.6	4.0
$C_{15:0}$	19.5	0.6	3.8	4.1
$C_{16:0}$	18.0	0.4	3.0	4.6
$C_{17:0}$	24.0	0.6	4.3	4.1
$C_{18:0}$	23.1	0.4	3.9	4.9

oleate and linoleate of phosphatidylcholine (Fig. 3b). As in mature lamina, palmitate was the most highly labelled fatty acid of phosphatidylglycerol (Fig. 3c).

Redistribution of ^{14}C among maize leaf lipids during pulse-chase experiments

Since the linolenate of monogalactosyl diacylglycerol was rapidly labelled in the immature maize

Fig. 4. Redistribution of label among the fatty acids of phosphatidylcholine and monogalactosyl diacylglycerol after the transfer of immature maize lamina from [^{14}C]acetate to [^{12}C]acetate

Phosphatidylcholine (a) and monogalactosyl diacylglycerol (b), labelled in the experiment described in Table 1, were purified and analysed as described in the Methods section. ▲, Palmitate; ○, oleate; ●, linoleate; Δ, linolenate.

Fig. 5. Time-course of labelling of the acyl moieties of glycerolipids in immature maize lamina by $^{14}\text{CO}_2$

Lamina strips from the emerging sixth leaf of maize seedlings were supplied with $^{14}\text{CO}_2$ under the conditions described in the Methods section. At each time shown, one strip was harvested and the radioactivity in the fatty acids of individual lipids determined. ○, Phosphatidylcholine; ▲, phosphatidylglycerol; Δ, monogalactosyl diacylglycerol; ●, digalactosyl diacylglycerol + sulpholipid + phosphatidylethanolamine.

lamina, we investigated the possibility that this might occur by transfer of label from the oleate and linoleate of phosphatidylcholine, as suggested by the long-term experiments of Roughan (1970) with mature pumpkin leaves. Lamina strips were fed with [^{14}C]acetate for 20 min, then transferred to [^{12}C]acetate and sampled at intervals during the subsequent 140 min (Table 1). To minimize the carry-over of labelled acetate, the portion of each lamina strip that had been immersed in the labelled solution and a 1 cm region above it was removed at the time of transfer. Incorporation of label into glycerolipids continued during the first 30 min after transfer, then ceased, suggesting that the uptake of [^{14}C]acetate into the lamina strips was faster than its incorporation into lipids. After transfer to [^{12}C]acetate there was a continuous loss of radioactivity counts from the oleate of phosphatidylcholine (Fig. 4a), which between 20 and 140 min represented a 5.5-fold decrease in specific radioactivity. The amount of label present in the oleic acid of monogalactosyl diacylglycerol (Fig. 4b) remained essentially constant during the experiment, and the increase in radioactivity in this lipid during the period in [^{14}C]acetate was attributable to an increased labelling of linoleate and linolenate. There was, however, little change in the distribution of label within the carbon chains of the oleate of phosphatidylcholine or the linolenate of monogalactosyl diacylglycerol subsequent to the transfer to unlabelled acetate, since both fatty acids were essentially uniformly labelled at the time of transfer

(Table 2). This redistribution of label among the different lipids in developing maize leaf lamina was very similar to, although faster than, that previously described by Roughan (1970).

Time-course of $^{14}\text{CO}_2$ incorporation into fatty acids of maize leaf lipids

The above results are consistent with the view that the acetate which entered the leaf lamina via the transpiration stream was incorporated initially into oleate and palmitate, and that these fatty acids were rapidly incorporated into phosphatidylcholine and phosphatidylglycerol respectively. If, however, the [^{14}C]acetate entered some acetate pools faster than others then our results would not truly reflect the routes of fatty acid formation.

We therefore determined the rate of entry of ^{14}C from photosynthetically fixed $^{14}\text{CO}_2$ into the fatty acids of individual lipids (Fig. 5). To prevent an excessive depletion of CO_2 in the photosynthesis apparatus during the relatively long period of the experiment the concentration of $^{14}\text{CO}_2$ initially supplied to the leaf lamina was 0.2% in air. Throughout the experiment the rates of ^{14}C incorporation into water-soluble products and lipids were essentially constant. The relative rates of labelling of the acyl moieties of individual lipids from $^{14}\text{CO}_2$ (Fig. 5) were similar to those obtained from [^{14}C]acetate. The fatty acids of phosphatidylcholine and phosphatidylglycerol were labelled at an essentially linear rate from zero time, whereas radioactivity entered the fatty acids of other lipids at accelerating rates throughout the experiment. The sequence of labelling of the individual fatty acids of phosphatidylcholine and monogalactosyl diacylglycerol was also similar to that obtained from [^{14}C]acetate (Fig. 2). As would be expected, a considerable amount of the ^{14}C was incorporated into the non-acyl portion of lipids, and, particularly in the galactolipids, this represented a large proportion of radioactivity in the lipid. After

feeding lamina with $^{14}\text{CO}_2$ for 13 min only 35% of the radioactivity in monogalactosyl diacylglycerol was present in the fatty acids compared with 72% in phosphatidylcholine fatty acids.

Intracellular location of rapidly labelled phosphatidylcholine and evidence for the transfer of fatty acids between lipids of different organelles

To identify the intracellular location of the phosphatidylcholine into which [^{14}C]acetate was rapidly incorporated, leaf lamina of maize was fed with labelled acetate and then homogenized and the homogenate fractionated by centrifugation (Table 3). At harvest, 48 and 34% of the total incorporated radioactivity was present in phosphatidylcholine and phosphatidylglycerol respectively. Of the radioactivity in phosphatidylcholine most was associated with 12000 g and 105000 g pellets, whereas the 2000 g pellet contained the greater proportion of the labelled phosphatidylglycerol. In the phosphatidylcholine of the different pellets the distribution of label among the fatty acids was almost identical (Table 4). The specific radioactivities, however, of oleate and linoleate of the 105000 g-pellet phosphatidylcholine was approximately twice that of the fatty acids from the phosphatidylcholine in the 2000 g pellet. As previously observed palmitate was the most heavily labelled fatty acid of phosphatidylglycerol and, as in phosphatidylcholine, the distributions of the fatty acids of this lipid in all fractions were similar. It was noted that the phosphatidylglycerol from the 2000 g and 105000 g pellets differed in the relative amounts of *trans*-3-hexadecenoic acid and palmitic acid. In chloroplasts the ratio of the masses of $\text{C}_{16:1}$ to $\text{C}_{16:0}$ was 1.3 and in the microsomal fraction 0.5. Although the above results suggest that the two rapidly labelled phospholipids were probably associated with different cell organelles, it was not possible by differential centrifugation, which only provides enrichment and not the complete separation of

Table 3. *Distribution of radioactivity among lipids present in different fractions prepared from mature maize leaf labelled with [^{14}C]acetate*

Lamina strips from maize leaf were fed with [$1\text{-}^{14}\text{C}$]acetate for 14 min and then homogenized; the homogenate was fractionated by differential centrifugation and the label in lipids, isolated from each pellet, determined as described in the Methods section. Samples were removed from each fraction for chlorophyll analysis before lipid extraction.

Lipid	$10^{-3} \times$ Radioactivity in individual lipids from each pellet (c.p.m.)		
	105000g	12000g	2000g
Phosphatidylcholine	127	105	31
Phosphatidylglycerol	28	59	95
Monogalactosyl diacylglycerol	7	9	19
Digalactosyl diacylglycerol + phosphatidylethanolamine + sulpholipid	11	11	22
Chlorophyll (μg)	0.1	0.5	2.8

Table 4. *Distribution and specific radioactivity of the fatty acids of phosphatidylcholine and phosphatidylglycerol in fractions prepared by differential centrifugation from mature maize leaf labelled with [¹⁴C]acetate*

The phosphatidylcholine and phosphatidylglycerol from the different pellets described in Table 3 were purified and their fatty acids analysed as described in the Methods section.

Percentage distribution of radioactivity in fatty acids of the individual lipids, and $10^{-2} \times$ specific radioactivity (c.p.m./ μ g) of each fatty acid (given in parentheses)

Pellet	Phosphatidylglycerol					Phosphatidylcholine			
	C _{16:0}	<i>trans</i> - Δ^3 -C _{16:1}	C _{18:1}	C _{18:2}	C _{18:3}	C _{16:0}	C _{18:1}	C _{18:2}	C _{18:3}
105000 g	55 (25.4)	2 (1.9)	38 (89)	4 (5.3)	1 (2.4)	5 (6.1)	64 (656)	27 (32.5)	2 (3.7)
12000 g	59 (29.8)	2 (0.9)	37 (66)	2 (1.5)	1 (0.4)	5 (5.7)	63 (486)	27 (17.1)	3 (3.4)
2000 g	54 (24.3)	2 (0.8)	39 (91)	3 (3.4)	1 (0.3)	6 (7.3)	64 (310)	28 (18.0)	2 (0.5)

Table 5. *Radioactivity in phosphatidylcholine and phosphatidylglycerol in fractions prepared from pea leaflets after [¹⁴C]acetate had been supplied for 20 min*

[¹⁴C]Acetate was supplied to pea shoots for 20 min, the leaflets were removed, homogenized and the homogenate centrifuged at 2000 g. The pellet was resuspended in a solution containing 0.33 M-sorbitol, layered above 0.4 M-sucrose and centrifuged again. Lipids were extracted separately from the sorbitol and sucrose layers, the pellet and the 2000 g supernatant and analysed. Details are given in the Methods section.

Fractions	Chlorophyll (μ g)	$10^{-3} \times$ Radioactivity (c.p.m.)	
		Phosphatidylcholine	Phosphatidylglycerol
0.33 M-Sorbitol	0.08	8.5	2.7
0.4 M-Sucrose	36	5.2	12.0
Pellet	217	13.1	25.2
2000 g supernatant	165	267.8	31.0

different particulate cell constituents, to determine whether the similar amounts of radioactivity in the phosphatidylcholine of the 12000 g and 105000 g pellets (Table 3) represented cross-contamination of the two pellets with a single labelled component or approximately equal labelling of different organelles.

Sucrose-density-gradient centrifugation afforded Lord *et al.* (1972) a much improved separation of cell organelles from castor-bean endosperm compared with differential centrifugation. Consequently, we applied their procedure (Kagawa *et al.*, 1973), in association with the method of Mackender & Leech (1974) for the partial purification of chloroplasts, to fractionate organelles from labelled pea leaflets (Table 5, Fig. 6). The sequence of labelling of the lipids of expanding pea leaflets has been found to be the same as that described above for maize (C. R. Slack & P. G. Roughan, unpublished work). Pea leaves were used in this fractionation experiment since only a brief homogenization period was required, compared with maize, to liberate sufficient cell constituents for analysis, and consequently less organelle disruption was expected during extraction. The 2000 g pellet contained 60% of the radioactivity

present in phosphatidylglycerol, the largest proportion of which was isolated with the more-intact chloroplasts that pelleted through 0.4 M-sucrose. The 2000 g supernatant, on the other hand, contained about 90% of the labelled phosphatidylcholine, which, on the sucrose density gradient (Fig. 6), was predominantly associated with a 280 nm-absorbing band at 25–29% (w/w) sucrose. Phosphatidylglycerol was about equally distributed between this band and the chlorophyll-containing zone. The band containing the highly labelled phosphatidylcholine occurred within the same density range as did microsomal fractions from the endosperms of castor bean (Lord *et al.*, 1972; Kagawa *et al.*, 1973) and water melon (Kagawa *et al.*, 1975).

Comparisons of the lipid composition of the membranes comprising the envelope and lamellae of spinach chloroplasts (Douce *et al.*, 1973) and broad-bean chloroplasts (Mackender & Leech, 1974) have shown that the envelope is relatively rich in phosphatidylcholine, whereas phosphatidylglycerol is the major phospholipid of the lamellae. The possibility existed, therefore, that the heavily labelled phosphatidylcholine, isolated in the high-speed pellets by

Fig. 6. Sucrose-density-gradient separation of particulate components in a 2000g supernatant prepared from expanding pea leaflets after they had been supplied with [^{14}C]acetate for 20 min

A sample of the 2000g supernatant described in Table 5 was fractionated on a sucrose density gradient as described in the Methods section. The distribution within the gradient of material with an absorbance at 280 nm was monitored (—), and the sucrose concentration (●), chlorophyll content (Δ) and labelled phosphatidylcholine (○) were determined in fractions collected from the gradient as described in the Methods section.

differential centrifugation and with the low-density membrane fraction on sucrose density gradients, could represent phosphatidylcholine of the chloroplast envelope, since the homogenization procedures used to disrupt the tissues may have stripped the envelopes from most of the chloroplasts. We investigated this possibility using spinach leaves (Table 6) in which the integrity of isolated chloroplasts could be assessed both by phase-contrast microscopy and by their ability to sustain O_2 evolution in the presence of only HCO_3^- . Chloroplasts in the 2000g pellets from the 1 h and 48 h harvests liberated O_2 in the presence of HCO_3^- at rates of 59 and $54 \mu\text{mol/h}$ per mg of chlorophyll respectively. These rates are about one-half of the maximum rates *in vivo* (Walker, 1971) and within the range of maximum chloroplast activities *in vitro* reported by Avron & Gibbs (1974) and Magalhaes *et al.* (1974). We assume, therefore, that a high proportion of the chloroplasts isolated in the 2000g pellets from both harvests were intact. Consequently, since very little of the total label present in phosphatidylcholine at the 1 h harvest (Table 6) was isolated in the 2000g pellet, it appeared very unlikely that the rapidly labelled phosphatidylcholine was associated with the chloroplast envelope. Very little chlorophyll was present in the 105000g pellets, but approx. 40% of the total chlorophyll released from

the leaf tissue pelleted at 12000g. The amount of radioactivity present in the lipids, per unit amount of chlorophyll isolated, at the 48 h harvest was only 90% of that present at the 1 h harvest. It is possible that this apparent loss of label could represent a dilution of the label initially present by leaf expansion and chloroplast division (Saurer & Possingham, 1970) during the 2 day period between harvests. The most pronounced change in the redistribution of label during the period between harvests was a large decrease in the amount of label in the oleate ($\text{C}_{18:1}$) of phosphatidylcholine in the 12000g and 105000g pellets and an increase in the radioactivity in the linolenate ($\text{C}_{18:3}$) of monogalactosyl diacylglycerol in 2000g and 12000g pellets. From the amount of chlorophyll in the latter fraction we assume that it contained about 40% of the plastid lamellae extracted. A small increase (not shown in Table 6) also occurred in the label present in the linolenate of digalactosyl diacylglycerol between harvests. It is also noteworthy that only in phosphatidylglycerol was there an appreciable and consistent loss of radioactivity from palmitate ($\text{C}_{16:0}$), between harvests, and the amount lost corresponded closely to that gained by the *trans*-3-hexadecenoate (*trans*- Δ^3 - $\text{C}_{16:1}$) of this lipid. This observation is consistent with the finding that palmitate is the precursor of *trans*-

observation that the labelled phosphatidylcholine in pea leaf homogenates was predominantly associated with a low-density membrane fraction on sucrose density gradients. Since chloroplasts appear to be the site of oleate synthesis (Stumpf & James, 1963), the present results imply that there is a transfer of oleate from chloroplasts to extrachloroplast membranes.

Although the redistribution of ^{14}C among the fatty acids of the glycerolipids of immature maize lamina after the removal of the supply of [^{14}C]acetate was much faster than that observed in mature pumpkin leaves (Roughan, 1970), the changes in labelling in the two tissues were similar, and the present results support Roughan's (1970) suggestion that the polyunsaturated fatty acids present in galactolipids are derived from the oleate of phosphatidylcholine. Similarly, the almost complete loss of label from the oleate of phosphatidylcholine in non-plastid membranes of spinach during a 48 h period after feeding with [^{14}C]acetate and the increase in radioactivity in the linolenate of monogalactosyl diacylglycerol suggest that fatty acids are transferred from non-chloroplast membranes to chloroplasts. Our present results provide no indication as to how this transfer is achieved. However, since the C_{18} fatty acids of phosphatidylcholine lost radioactivity, but palmitate did not, it appears that the transfer of specific fatty acids is involved.

Very little ^{14}C accumulated in the linolenate of phosphatidylcholine compared with that in the linolenate of the galactolipids. This finding, together with the more rapid labelling of the linolenate of phosphatidylcholine than that of monogalactosyl diacylglycerol and the movement of label into, then out of, the linoleate of this phospholipid during a chase in unlabelled acetate, suggests that linoleate could be the major fatty acid donated by phosphatidylcholine. An alternative possibility is that phosphatidylcholine is associated with linolenate formation but that this fatty acid is transferred rapidly from the phospholipid to galactolipids. The former hypothesis is attractive since linoleate in phosphatidylcholine appears to be the product of microsomal oleate desaturases from a number of organisms (Baker & Lynen, 1971; Talamo *et al.*, 1973) and of a desaturase isolated in a chloroplast-containing fraction from *Chlorella* (Gurr *et al.*, 1969). The concomitant linear rates of labelling of the oleate and linoleate of phosphatidylcholine, however, do not immediately suggest a precursor-product relationship, since one would expect that the oleate would need to be saturated with ^{14}C before a constant rate of labelling of the linoleate could occur. A similar, though less pronounced, discrepancy was reported in the labelling of linoleate in *Chlorella* by supplied [^{14}C]oleate (Gurr *et al.*, 1969). Gurr & Brawn (1970) found that oleate was incorporated into several forms of phosphatidylcholine in

Chlorella; the rates of incorporation and desaturation differed between the various forms. A similar selectivity in the formation and desaturation of certain oleate-containing forms of phosphatidylcholine in maize could explain the labelling patterns observed.

The very similar distribution of label observed among the carbon atoms of the oleate of phosphatidylcholine and the linolenate of monogalactosyl diacylglycerol is consistent with the sequential desaturation of oleate to linoleate and then to linolenate, and agrees with the data of Tremolieres & Mazliak (1974). Our results, however, are not consistent with the suggestion (Kannangara *et al.*, 1973; Jacobson *et al.*, 1973) that linolenate is formed by chain elongation of a $\text{C}_{12:3}$ fatty acid.

Implicit in the above hypothesis is that the conversion of oleate into linoleate is associated with phosphatidylcholine of a microsomal membrane and that the desaturation of linoleate to linolenate occurs elsewhere in the cell, possibly in the chloroplasts. A similar scheme, based on the ability of pea microsomal fractions to desaturate oleoyl-CoA, and of pea chloroplasts to desaturate linoleate, has been proposed by Tremolieres & Mazliak (1974). Confirmation of these views, however, must await the characterization of oleate and linoleate desaturases and the identification of a mechanism for linoleoyl transfer between organelles.

We thank Mrs. J. Terpstra for excellent technical assistance during this work.

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