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The acylation of *sn*-glycerol 3-phosphate and the metabolism of phosphatidate in microsomal preparations from the developing cotyledons of safflower (*Carthamus tinctorius* L.) seed

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Microsomal preparations from the developing cotyledons of safflower (*Carthamus tinctorius*) catalysed the acylation of *sn*-glycerol 3-phosphate in the presence of acyl-CoA. The resulting phosphatidate was further utilized in the synthesis of diacyl- and tri-acylglycerol by the reactions of the so-called 'Kennedy pathway' [Kennedy (1961) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **20**, 934–940]. Diacylglycerol equilibrated with the phosphatidylcholine pool when glycerol backbone, with the associated acyl groups, flowed from phosphatidate to triacylglycerol. The formation of diacylglycerol from phosphatidate through the action of a phosphatidate phosphohydrolase (phosphatidase) was substantially inhibited by EDTA and, under these conditions, phosphatidate accumulated in the microsomal membranes. The inhibition of the phosphatidase by EDTA was alleviated by Mg^{2+}. The presence of Mg^{2+} in all incubation mixtures stimulated quite considerably the synthesis of triacylglycerol *in vitro*. Microsomal preparations incubated with acyl-CoA, *sn*-glycerol 3-phosphate and EDTA synthesized sufficient phosphatidate for the reliable analysis of its intramolecular fatty acid distribution. In the presence of mixed acyl-CoA substrates the *sn*-glycerol 3-phosphate was acylated exclusively in position 1 with the saturated fatty acids, palmitate and stearate. The polyunsaturated fatty acid linoleate was, however, utilized largely in the acylation of position 2 of *sn*-glycerol 3-phosphate. The affinity of the enzymes involved in the acylation of positions 1 and 2 of *sn*-glycerol 3-phosphate for specific species of acyl-CoA therefore governs the non-random distribution of the different acyl groups in the seed triacylglycerols. The acylation of *sn*-glycerol 3-phosphate in position 1 with saturated acyl components also accounts for the presence of these groups in position 1 of *sn*-phosphatidylcholine through the equilibration of diacylglycerol with the phosphatidylcholine pool, which occurs when phosphatidate is utilized in the synthesis of triacylglycerol. These results add further credence to our previous proposals for the regulation of the acyl quality of the triacylglycerols that accumulate in developing oil seeds [Stymne & Stobart (1984) *Biochem. J.* **220**, 481–488; Stobart & Stymne (1985) *Planta* **163**, 119–125].

Recently we proposed a working model that explains the C_{18}-unsaturated-fatty-acid composition of the triacylglycerols that accumulate in the developing cotyledons of oil-rich seeds such as sunflower (Stymne & Stobart, 1984*a*), safflower (Stobart & Stymne, 1985) and linseed (Stymne & Stobart, 1985). It is well established that the desaturation of oleate (octadec-9-enoic acid) to linoleate (octadec-9,12-dienoic acid) (Stymne & Appelqvist, 1978; Slack *et al.*, 1979; Stobart *et al.*, 1983) and, possibly linolenate (octadec-9,12,15-trienoic acid) (Stymne & Appelqvist, 1980; Browse & Slack, 1981) occurs in position 2 of microsomal *sn*-phosphatidylcholine. A major problem has been to account for the entry of oleate into phosphatidylcholine and to establish how the C_{18}-polyunsaturated-fatty-acid products are made available for

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the synthesis of triacylglycerols. Roughan & Slack (1982) suggest that the phosphatidylcholine gives rise to polyunsaturated species of diacylglycerols that are further acylated to yield triacylglycerol. We have shown in microsomal preparations, however, that the oleate in oleoyl-CoA equilibrates with linoleate in position 2 of *sn*-phosphatidylcholine (Stymne *et al.*, 1983; Stobart *et al.*, 1983; Stymne & Stobart, 1984*a*) and that this is probably catalysed by the forward and reverse reactions of an acyl-CoA : lysophosphatidylcholine acyltransferase enzyme working in concert (Stymne & Stobart, 1984*b*). The acyl exchange returns linoleate to the acyl-CoA pool where it is selectively utilized in the acylation of *sn*-glycerol 3-phosphate to give phosphatidate (Stymne *et al.*, 1983; Stobart *et al.*, 1983), which in its turn is converted into diacyl- and tri-acylglycerol by the reactions of the 'Kennedy' (1961) pathway (Stobart & Stymne, 1985). We have also demonstrated an equilibration between diacylglycerol and phosphatidylcholine, possibly catalysed by a CDP : diacylglycerol cholinephosphotransferase (Slack, 1983; Slack *et al.*, 1983), which occurs when glycerol backbone flows from phosphatidate to triacylglycerol (Stymne & Stobart, 1984*a*). The equilibration of diacylglycerol with phosphatidylcholine may give further opportunity to desaturate oleate and so brings about a continual enrichment of the glycerol backbone with C_{18} polyunsaturated fatty acids (Stobart & Stymne, 1985).

Any scheme presented for the biosynthesis of the triacylglycerols in oil-rich seeds has to account, however, for the intramolecular distribution of the acyl species in the complex lipids (Hilditch & Williams, 1964). The triacyl-*sn*-glycerols of safflower (*Carthamus tinctorius* L.), for example, contain relatively high quantities of the saturated fatty acids, palmitate (hexadecanoic acid) and stearate (octadecanoic acid) at position 1, with none, or very little, in position 2 (Ichihara & Noda, 1980). We proposed, therefore, that the properties of the enzymes that acylate *sn*-glycerol 3-phosphate may account for the distribution of particular fatty acid species in the triacylglycerols (Stobart & Stymne, 1985). The positional acylation of *sn*-glycerol 3-phosphate should also reflect the fatty acids in phosphatidylcholine due to the equilibration of diacylglycerols with this phospholipid during triacylglycerol synthesis. A study of the selective utilization of the saturated fatty acids, palmitate and stearate, in the synthesis of lipids in microsomal preparations from developing oilseeds should therefore provide a means whereby our suggestions could be further verified. We now present evidence which strongly supports our proposals for the regulation of the acyl quality of the triacylglycerols in developing oilseeds.

Materials and methods

Chemicals

1-^{14}C-labelled fatty acids and L-*sn*-[U-^{14}C]glycerol 3-phosphate were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin (fraction V, fatty-acid-free), EDTA, CoASH, L-*sn*-glycerol 3-phosphate, phospholipase A_2 [from Indian-cobra (*Naja naja*) venom] and various fatty acids were purchased from Sigma.

[1-^{14}C]Palmitoyl-CoA (hexadecanoyl-CoA) (sp. radioactivity 2746d.p.m./nmol), [1-^{14}C]stearoyl-CoA (octadecanoyl-CoA) (sp. radioactivity 1640d.p.m./nmol), [1-^{14}C]oleoyl-CoA (octadec-9-enoyl-CoA) (sp. radioactivity 2836d.p.m./nmol), unlabelled oleoyl-CoA, [1-^{14}C]linoleoyl-CoA (octadec-9,12-dienoyl-CoA) (sp. radioactivity 2340d.p.m./nmol) and [1-^{14}C]linolenoyl-CoA (octadec-9,12,15-trienoyl-CoA) (sp. radioactivity 1701d.p.m./nmol) were synthesized from their mixed anhydrides as described by Sanchez *et al.* (1973). The radioactive acyl-CoA species had a chemical and radiochemical purity of more than 98% as determined by t.l.c., g.l.c. and radio-g.l.c.

Microsomal preparations

Safflower (*Carthamus tinctorius* L.) plants, var. Gila (a high-linoleate-oil variety), were grown from seed in a 16h photoperiod at 28°C with a light intensity of 20klux and a 8h night at 22°C. The plants were hand-pollinated and the seeds were harvested 14–18 days after flowering and the cotyledons were removed and stored on ice. All further procedures were carried out at 1–4°C. The cotyledons were ground in a mortar with 2 parts (w/v) of 0.1M-potassium phosphate buffer, pH7.2, containing 0.1% bovine serum albumin and 0.33M-sucrose. The homogenate was filtered through a double layer of Miracloth, diluted 5-fold with fresh grinding medium and centrifuged at 20000*g* for 20min. The supernatant was filtered through Miracloth and centrifuged at 105000*g* for 90min. The resulting microsomal pellet was resuspended in 0.1M-potassium phosphate buffer, pH7.2, and stored at −70°C until required.

Enzyme assays

All assays were done at 30°C with constant shaking. Incubation mixtures contained 10mg of bovine serum albumin, substrates and cofactors dissolved in 0.1M-phosphate buffer, pH7.2, at the concentrations stated in the Tables and Figures, and the microsomal preparation adjusted to a final volume of 1ml with 0.1M-phosphate buffer.

Analytical procedures

Reactions were terminated by the addition of

0.15M-acetic acid and the lipids extracted in a modification of the medium described by Bligh & Dyer (1959). The lower chloroform phase, which contains the complex lipids and free fatty acids, was removed and evaporated to dryness under N_2. The residue was dissolved in a small volume of chloroform and the polar and neutral lipids were purified by t.l.c. on precoated silica-gel plates (Merck; silica-gel 60) with chloroform/methanol/acetic acid/water (170:30:20:7, by vol.) or hexane/diethyl ether/acetic acid (70:30:1, by vol.) respectively. Lipid areas, located by lightly staining with I_2 vapour, were removed from the plates and either assayed for radioactivity or methylated *in situ* with methanolic 2.5% (w/w) HCl (Kates, 1964) for the analysis of fatty acids. The fatty acid methyl esters were analysed by g.l.c. with a glass column (2m×2mm) containing 10% BDS on Chromosorb W (HP, 80–100 mesh) and quantified by using methylheptadecanoic acid as an internal standard or analysed by radio-g.l.c. for the determination of the radioactivity in the fatty acids.

Positional analysis of the fatty acids in phosphatidylcholine and phosphatidate was performed by the treatment of the lipids with phospholipase A_2. The phospholipids were eluted from the gel in 2.25ml of methanol/chloroform (2:1, v/v) for 18h at 4°C. The eluates were evaporated to dryness under N_2 and the lipids redissolved in 1ml of diethyl ether. The lipid samples in diethyl ether were sonicated for 10min at 0°C with 1ml of 0.1M-borate buffer, pH8.9 (Van-Golde & Van-Deenen, 1966) and then 25 units of phospholipase A_2 were added. Enzymic hydrolysis was allowed to proceed at 25°C for 80min with vigorous mixing. After evaporation of the diethyl ether the products were extracted in 1ml of acidified butan-1-ol saturated with water [butan-1-ol/water/acetic acid, 1:0.95:0.5 (by vol.); modified from that described by Bjerve *et al.* (1974)], followed by a further 1ml of butan-1-ol. After centrifugation of the combined extracts, the butan-1-ol phase was removed and evaporated to dryness under nitrogen. The lipid residue was dissolved in chloroform and the lysophospholipids and non-esterified fatty acids were separated in the t.l.c. system for polar lipids (see above). Methylation was accomplished using methanolic HCl. The method for the positional analysis for the phosphatidate was checked by using egg phosphatidate (Sigma) and gave equimolar quantities of fatty acids from positions 1 and 2.

Lipid samples were assayed for radioactivity in PCS (Amersham/Searle)/xylene (2:1, v/v) scintillant in a Beckman LS-230 liquid-scintillation counter with an efficiency of 94% for ^{14}C. All radioactivity counts were corrected for background and quenching.

Results

Effect of Mg^{2+} and EDTA on the incorporation of sn-[U-^{14}C]glycerol 3-phosphate into lipids

Microsomal preparations from the developing cotyledons of safflower were incubated with *sn*-[^{14}C]glycerol 3-phosphate (sp. radioactivity 3880d.p.m./nmol) and oleoyl-CoA in the presence of either Mg^{2+} or EDTA. At regular intervals the [^{14}C]glycerol in the complex lipids was determined (Fig. 1). Incubation with EDTA for 20min resulted in some 30% more radioactive glycerol in phosphatidate than found in the control incubations. On the other hand, the incorporation of glycerol in phosphatidate, in incubations with Mg^{2+} for 20min, was 50% of the control value. The subsequent addition of Mg^{2+} to the microsomal preparations, which had been incubated with EDTA for 20min, brought about a rapid depletion in the radioactive phosphatidate that had accumulated. The effects of EDTA and Mg^{2+} on the activity of the phosphatidate phosphohydrolase reflected the amounts of radioactive glycerol in the other lipids. EDTA decreased and Mg^{2+} increased the radioactivity in phosphatidylcholine and diacyl- and triacyl-glycerol. The addition of Mg^{2+} to incubations with EDTA overcame the inhibitory affects observed with EDTA alone.

Utilization of [^{14}C]acyl-CoA in the acylation of sn-glycerol 3-phosphate

Microsomes, incubated with EDTA, *sn*-glycerol 3-phosphate and acyl-CoA, accumulate *sn*-phosphatidate in sufficient quantity for purification and the analysis of the acyl components at positions 1 and 2. Microsomal preparations (equivalent to 150nmol of phosphatidylcholine) were therefore incubated with mixed species of [^{14}C]-acyl-CoA (40nmol of each acyl-CoA substrate), *sn*-glycerol 3-phosphate (400nmol) and EDTA (10mM). After 20min of incubation the ^{14}C-labelled fatty acids in the phosphatidate and phosphatidylcholine were 31% and 43% respectively of the total radioactivity in the lipid fraction. All the radioactive acyl-CoA species were utilized, to varying extents, in the acylation of *sn*-glycerol 3-phosphate (Table 1). Palmitate was incorporated most efficiently, and this amounted to some 40% of the total radioactive fatty acids in the phosphatidate. The other acyl-CoA species were utilized in the acylation of *sn*-glycerol 3-phosphate in the order: linoleoyl > oleoyl > stearoyl > linolenoyl. The intramolecular distribution of the radioactive acyl groups present at C-1 and -2 of the *sn*-phosphatidate is given in Table 2. The radioactive palmitate and stearate were present exclusively at position 1, whereas linoleate and linolenate were largely associated with position 2. Oleate, on the

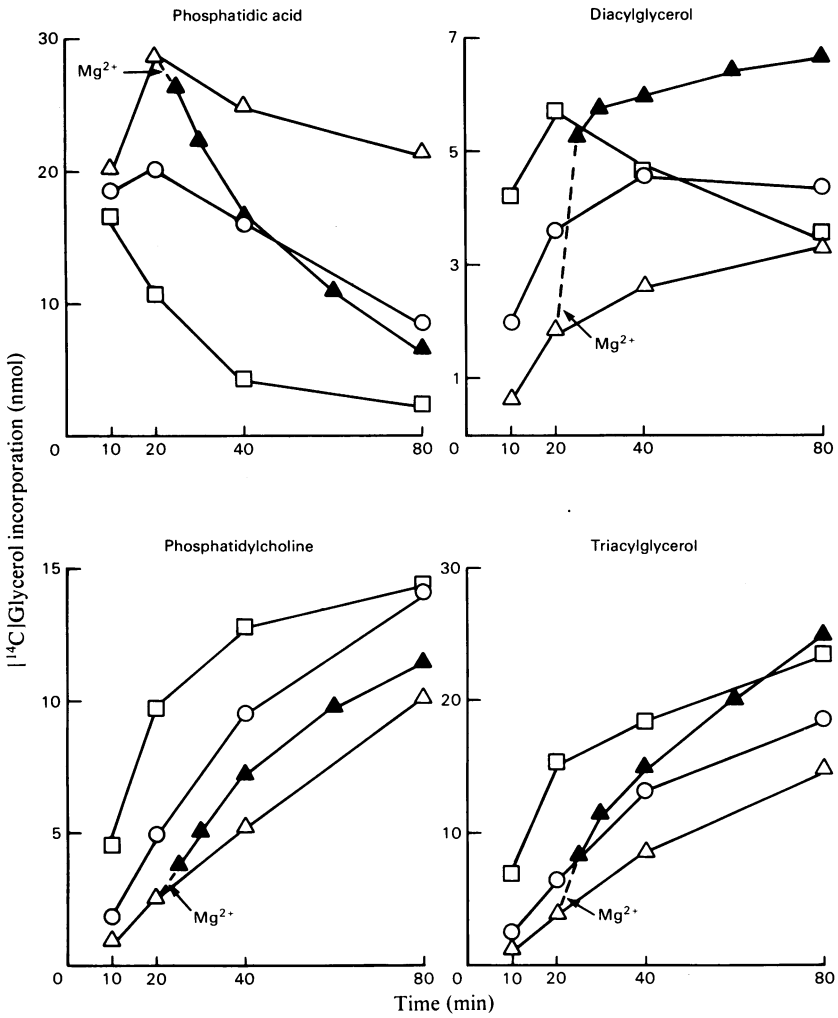

Fig. 1. *Effect of EDTA and Mg^{2+} on the utilization of sn-[^{14}C]glycerol 3-phosphate in safflower microsomal preparations* Microsomes (equivalent to 150nmol of phosphatidylcholine) were incubated with *sn*-[^{14}C]glycerol 3-phosphate (200nmol) and oleoyl-CoA (200nmol) in the presence of either EDTA (10mM; △) or $MgCl_2$ (20mM; □). Microsomal preparations were also incubated with the substrates and EDTA for 20min before the addition of Mg^{2+} (▲). Control treatments were in buffer only (○). At regular intervals the radioactivity in the complex lipids was determined. The results are the means for two separate incubations.

other hand, was incorporated most efficiently into position 1, where it represented over 90% of that particular fatty acid present in the *sn*-phosphatidate. The results show that the enzymes which acylate the *sn*-glycerol 3-phosphate in the presence of mixed acyl-CoA substrates exclude completely the saturated fatty acids from C-2. The polyunsaturated C_{18} fatty acids, however, are used preferentially in the acylation of position 2 of *sn*-glycerol 3-phosphate. It should be noted that the differing amounts of the total ^{14}C-labelled fatty acids found in position 1 and 2 (Table 2) is due to a dilution of the specific radioactivity of the linoleate by acyl exchange (see below), and hence the acylation of *sn*-glycerol 3-phosphate with linoleate is considerably greater than indicated by the results presented in Table 2.

Because of the total exclusion of the saturated fatty acids from position 2 of *sn*-phosphatidate during incubation with mixed species of acyl-CoA, it was considered worthwhile to investigate the acylation of *sn*-glycerol 3-phosphate with palmitoyl-CoA and stearoyl-CoA when presented to the microsomal preparations as single substrates.

Table 1. *Incorporation of ^{14}C-labelled fatty acids into phosphatidate in microsomes from developing safflower cotyledons incubated with a mixed [^{14}C]acyl-CoA substrate and sn-glycerol 3-phosphate*

Microsomes, equivalent to 150nmol of phosphatidylcholine, were incubated with [^{14}C]acyl-CoA (200nmol; 40nmol of each acyl-CoA), *sn*-glycerol 3-phosphate (400nmol) and EDTA (10mM). After 20min incubation the ^{14}C-labelled fatty acids in the phosphatidate were determined. For experimental details, see the Materials and methods section. Results are means ± S.D. for four incubations.

^{14}C-labelled fatty acid	Amount in phosphatidate (nmol)	(%)
Palmitate	10.6±0.4	37
Stearate	3.4±0.1	12
Oleate	5.4±0.2	19
Linoleate	7.8±0.3	27
Linolenate	1.5±0.1	5

Table 2. *Positional distribution of ^{14}C-labelled fatty acids in phosphatidate in microsomes from developing safflower cotyledons incubated with a mixed [^{14}C]acyl-CoA substrate and sn-glycerol 3-phosphate*

Microsomes, equivalent to 150nmol of phosphatidylcholine, were incubated with [^{14}C]acyl-CoA (200nmol; 40nmol of each acyl-CoA), *sn*-glycerol 3-phosphate (400nmol) and EDTA (10mM). After 20min incubation the phosphatidate was purified, treated with phospholipase A_2, and the radioactivity in the non-esterified fatty acids and in lysophosphatidate determined. For experimental details, see the Materials and methods section. Results are ±S.D. for four analyses.

^{14}C-labelled fatty acid	Positional distribution in *sn*-phosphatidate: Position 1 (nmol)	Position 1 (%)	Position 2 (nmol)	Position 2 (%)
Palmitate	10.6±0.4	100	0	0
Stearate	3.4±0.1	100	0	0
Oleate	4.1±0.6	93	0.3±0.1	7
Linoleate*	2.2±0.2	29	5.5±0.5	71
Linolenate	0.5±0.1	35	1.0±0.1	65

* It should be noted that the incorporation of linoleate into phosphatidate is an underestimate. This is due to the dilution of the specific radioactivity of the linoleoyl-CoA by the non-labelled linoleate that arises by acyl exchange between the acyl-CoA and position 2 of *sn*-phosphatidylcholine.

The results (Table 3) show that both species of acyl-CoA were efficiently incorporated into phosphatidate and phosphatidylcholine. Analysis of the acyl composition of the synthesized phosphatidate in incubations with either acyl-CoA showed that the major fatty acid present was linoleate (Table 4). The amount of linoleate present in the phosphatidate was similar to the quantity of radioactive palmitate or stearate recovered in phosphatidylcholine (see Table 3). The non-radioactive linoleate, which was utilized in the acylation of *sn*-glycerol 3-phosphate, is therefore equivalent to the amount of saturated radioactive fatty acid that had been exchanged for the major acyl group (i.e. linoleate) present in position 2 of *sn*-phosphatidylcholine. The acyl exchange makes linoleoyl-CoA available, and this is preferentially used in the acylation of position 2 of *sn*-glycerol 3-phosphate. The intramolecular distribution of radioactive palmitate and stearate in *sn*-phosphatidate (Table 5) shows that, although most of the saturated fatty acids were in position 1, some had been utilized in the acylation of position 2 of *sn*-glycerol 3-phosphate. The amount of the saturated fatty acids in position 2 of *sn*-phosphatidate is determined by the availability of the non-radioactive linoleoyl-CoA, through acyl exchange, in the acyl-CoA pool. Hence the small quantities of palmitate (20%) and stearate (5%) in position 2 of *sn*-phosphatidate were most probably used in the acylation of position 2 of *sn*-glycerol 3-phosphate before sufficient linoleate from phosphatidylcholine had entered the acyl-CoA pool.

The movement of linoleate to phosphatidate from phosphatidylcholine via acyl-CoA was confirmed in a pulse–chase experiment (Table 6). Microsomal preparations (equivalent to 95nmol of phosphatidylcholine) were incubated with 26nmol of [^{14}C]linoleoyl-CoA in the presence of EDTA. After 15min incubation, almost all radioactive linoleate was recovered in the chloroform-soluble lipids, of which 80% was present in the phosphatidylcholine. Very little radioactivity was present in the phosphatidate. Membrane preparations, which contained the [^{14}C]linoleoylphosphatidylcholine, were then incubated with non-radioactive oleoyl-CoA and *sn*-glycerol 3-phosphate for a further 10 and 20min. Control incubations without further additions were made for the same periods. At 10 and 20min after the addition of oleoyl-CoA and *sn*-glycerol 3-phosphate, 53 and 65% of the radioactivity respectively had been lost from the phosphatidylcholine. Almost half of the radioactive linoleate chased from the phosphatidylcholine was recovered in phosphatidate, which had accumulated in the presence of EDTA. The remaining radioactive linoleate that was removed from the phosphatidylcholine was not present in any of the other chloroform-soluble lipids and was almost certainly lost to the aqueous methanol phase as acyl-CoA during the extraction of lipids from the microsomal membranes (Stobart *et al.*,

Table 3. *Incorporation of ^{14}C-labelled fatty acid into phosphatidate and phosphatidylcholine in microsomes from developing safflower cotyledons incubated with either [^{14}C]palmitoyl-CoA or [^{14}C]stearoyl-CoA*

Microsomes, equivalent to 150nmol of phosphatidylcholine, were incubated with either [^{14}C]palmitoyl-CoA (200nmol) or [^{14}C]stearoyl-CoA (200nmol), *sn*-glycerol 3-phosphate (400nmol) and EDTA (10mM). After 20min incubation the ^{14}C-labelled fatty acid in phosphatidate and phosphatidylcholine was determined. For experimental details, see the Materials and methods section. The results shown are from a representative experiment.

	Incorporation of ^{14}C-labelled fatty acid					
	Phosphatidate		Phosphatidylcholine		Remaining lipids	
[^{14}C]Acyl-CoA substrate	(nmol)	(%)	(nmol)	(%)	(nmol)	(%)
Palmitate	19.4	27	24.4	34	27.8	39
Stearate	19.6	25	30.5	39	27.3	36

Table 4. *Fatty acid compositions of phosphatidate in microsomes from developing safflower cotyledons incubated with either [^{14}C]palmitoyl-CoA or [^{14}C]stearoyl-CoA*

The phosphatidate in the experiment described in Table 3 was transmethylated and its fatty acid content determined. For experimental details, see the Materials and methods section.

	Phosphatidate composition							
Fatty acid ...	Palmitate		Stearate		Oleate		Linoleate	
[^{14}C]Substrate	(nmol)	(%)	(nmol)	(%)	(nmol)	(%)	(nmol)	(%)
Palmitoyl-CoA	20.8	41	0	0	0	0	29.7	59
Stearoyl-CoA	3.4	7	15.1	32	0	0	29.0	61

Table 5. *Positional distribution of ^{14}C-labelled fatty acid in phosphatidate in microsomes from developing safflower cotyledons incubated with either [^{14}C]palmitoyl-CoA or [^{14}C]stearoyl-CoA*

The phosphatidate in the experiment described in Table 3 was treated with phospholipase A_2 and the radioactivity in the non-esterified fatty acids and lysophosphatidate determined. For experimental details, see the Materials and methods section.

	Positional distribution of ^{14}C-labelled fatty acid in *sn*-phosphatidate (%)	
[^{14}C]Substrate	Position 1	Position 2
Palmitoyl-CoA	80	20
Stearoyl-CoA	95	5

1983). A small decrease in the radioactive linoleate in phosphatidylcholine in the control incubations was also observed (10 and 21% after 10 and 20min incubation respectively) without a concomitant increase in the radioactive phosphatidate.

Incorporation of [^{14}C]acyl-CoA into phosphatidylcholine in the absence of sn-glycerol 3-phosphate

The experiment above demonstrated that the saturated fatty acids were restricted to position 1 of *sn*-phosphatidate when presented in a mixed substrate together with polyunsaturated fatty acid-CoA esters (Table 2). It was therefore of interest to carry out experiments on the utilization of the saturated fatty-acid CoA species in acyl exchange. The incorporation of [^{14}C]acyl-CoA, as mixed and single substrates, into phosphatidylcholine through acyl exchange only (i.e. in the absence of *sn*-glycerol 3-phosphate) was therefore investigated. Microsomal preparations were incubated with free CoASH (CoA stimulates acyl exchange; Stymne & Stobart, 1984*b*) and [^{14}C]acyl-CoA (mixed substrates: 160nmol, containing 32nmol of each acyl-CoA species; single substrate: 160nmol of each acyl-CoA), and after 10min the radioactive fatty acids in phosphatidylcholine were determined (Table 7). The results with the mixed acyl-CoA substrate show that oleate and linoleate were particularly well utilized, whereas the saturated fatty acids, palmitate and stearate, were completely selected against. The results indicate that saturated fatty acids are perhaps only incorporated into phosphatidylcholine through acyl exchange when the unsaturated C_{18} substrates are present in limiting concentrations. This was confirmed in experiments with single acyl-CoA substrates (Table 7), where relatively small quantities of the saturated fatty acids were incorporated into phosphatidylcholine. Almost all of the radioactive fatty acids in *sn*-phosphatidylcholine were associated with C-2 (results not shown).

Table 6. *Metabolism of [^{14}C]linoleoyl phosphatidylcholine in the presence and absence of oleoyl-CoA and glycerol 3-phosphate (Gro3P) in microsomal preparations from developing safflower cotyledons*

Microsomes, equivalent to 95nmol of phosphatidylcholine, were incubated with 26nmol of [^{14}C]linoleoyl-CoA, 10mM-EDTA and 200nmol of CoASH for 15min, followed by a 10 and 20min incubation, either in the absence or the presence of oleoyl-CoA (200nmol) and glycerol 3-phosphate (400nmol). The radioactive distribution between the lipids was determined at the different incubation periods and under different treatments. For the experimental details, see Materials and methods section. Results are means ± S.D. for two incubations.

Incubation time (min)	Treatment	[^{14}C]Linoleate (nmol) in: Chloroform-soluble lipids	Phosphatidylcholine	Phosphatidate
15	—	27.6 ± 0.9	21.6 ± 1.0	0.6 ± 0.0
25	—	25.9 ± 1.4	19.5 ± 1.1	0.6 ± 0.0
35	—	23.9 ± 2.1	17.0 ± 1.5	0.5 ± 0.0
15 + 10	— / Gro3*P* + oleoyl-CoA	21.0 ± 1.9	10.1 ± 0.4	5.2 ± 0.7
15 + 20	— / Gro3*P* + oleoyl-CoA	20.9 ± 0.2	7.6 ± 0.3	6.7 ± 0.6

Table 7. *Incorporation of ^{14}C-labelled fatty acids into phosphatidylcholine in microsomes from developing safflower cotyledons incubated with mixed and single [^{14}C]-acyl-CoA substrates*

Microsomes, equivalent to 74nmol of phosphatidylcholine, were incubated with [^{14}C]acyl-CoA (mixed substrate, 160nmol total, 32nmol of each acyl-CoA; single substrate, 160nmol of acyl-CoA) and CoASH (200nmol). After 10min incubation the ^{14}C-labelled fatty acids in phosphatidylcholine were determined. For experimental details, see the Materials and methods section. Results are means ± S.D. for two incubations.

[^{14}C]Acyl group	^{14}C-labelled fatty acids incorporated into phosphatidylcholine: Single substrate (nmol)	Mixed substrate (nmol)	(%)
Palmitate	4.3 ± 0.1	0	0
Stearate	5.4 ± 0.3	0	0
Oleate	29.6 ± 0.7	11.7 ± 0.2	47
Linoleate	30.6 ± 0.1	9.8 ± 0.1	39
Linolenate	15.8 ± 0.2	3.7 ± 0.2	14

Fatty acid composition of endogenous microsomal phosphatidylcholine and phosphatidate

Our suggested model for the production of triacylglycerols in oilseeds envisages that the glycerol backbone with the associated acyl groups equilibrates with the phosphatidylcholine during the operation of the Kennedy pathway (see the introduction). Hence the specific selection of acyl-CoA species exhibited by the *sn*-glycerol 3-phosphate-acylating enzymes should regulate the saturated-fatty-acid composition of the endogenous phosphatidate. Linoleate is synthesized from oleate that is esterified to phosphatidylcholine. Oleate can enter phosphatidylcholine either by acyl exchange at position 2 or by diacylglycerol ⇄ phosphatidylcholine interconversion. Therefore a comparison between the oleate/linoleate ratios in endogenous phosphatidate and phosphatidylcholine would help to reveal the relative importance of the acyl exchange and diacylglycerol⇄phosphatidylcholine interconversion for the influx of oleate into phosphatidylcholine. Sufficient microsomal phosphatidate was obtained for the analysis of the fatty acids, and its composition was found to be almost identical with that of phosphatidylcholine (Table 8). The major fatty acid in both phospholipids was linoleate. Oleate and stearate were also present in small amounts, whereas palmitate represented over 12% of the total fatty acid complement.

Discussion

Microsomal preparations from the developing seed cotyledons of safflower catalysed the acylation of *sn*-glycerol 3-phosphate to yield phosphatidate (1,2-diacyl-*sn*-glycerol 3-phosphate). The phosphatidate was further metabolized to triacylglycerols by the reactions of the Kennedy (1961) pathway. EDTA was found to inhibit substantially the activity of the phosphatidate phosphohydrolase (EC 3.1.3.4) and, under these conditions, phosphatidate accumulated in the incubation mixtures. The inhibitory effects of EDTA on the activity of the phosphohydrolase was overcome with Mg^{2+}. In fact the addition of Mg^{2+} to reaction mixtures not containing EDTA enhanced the rate of triacylglycerol production. Microsomal preparations from developing oilseeds appear to be deficient, to some extent, in bivalent cations, and

Table 8. *Fatty acid composition of endogenous phosphatidate and phosphatidylcholine in microsomes from developing safflower cotyledons*

Microsomal preparations were extracted in Bligh & Dyer (1959) medium and the purified phospholipids transmethylated for fatty acid analysis. For experimental details, see the Materials and methods section. Results are means ± S.D. for the analysis of three microsomal extracts.

	Composition (mol/100 mol)			
Fatty acid ...	Palmitate	Stearate	Oleate	Linoleate
Phosphatidate	13 ± 0.6	2 ± 0.1	2 ± 0.1	83 ± 0.7
Phosphatidylcholine	12 ± 0.3	3 ± 0.2	1 ± 0.1	84 ± 0.4

hence much greater triacylglycerol synthesis can be achieved *in vitro* in the presence of Mg^{2+}. No attempts were made to test the effect of other bivalent cations on the activity of the microsomal phosphohydrolase. It is noteworthy that many of the phosphohydrolases from animal sources are also strongly stimulated by Mg^{2+} (Hosaka *et al.*, 1975; Call & Williams, 1973; Lamb & Fallon, 1974; Sturton & Brindley, 1980), and quite recently the purified enzyme from *Saccharomyces* was reported to have an absolute requirement for this cation (Hosaka & Yamashita, 1984). The phosphohydrolase of the chloroplast inner membrane, however, appears to be different in its properties compared with the enzyme located in the endoplasmic reticulum, and is inhibited by Mg^{2+} (Block *et al.*, 1983).

Microsomal preparations from the oilseeds accumulate phosphatidate in incubation mixtures containing the appropriate substrates and EDTA. Under these conditions the *sn*-phosphatidate was present in sufficient quantity for the accurate positional analysis of the acyl groups esterified to C-1 and -2. It was found that the *sn*-glycerol 3-phosphate-acylating enzymes were selective towards the acyl-CoA substrates and, in the presence of mixed acyl-CoA species, the saturated fatty acids, palmitate and stearate, were utilized exclusively in the acylation of position 1. Linoleate, on the other hand, was the preferred acyl-CoA substrate in the acylation of position 2 of *sn*-glycerol 3-phosphate. It is also of interest that oleate is more efficiently incorporated into position 1. The oilseed *sn*-glycerol 3-phosphate acyltransferase system appears to be different in its acyl selectivity properties compared with the enzymes present in the membranes of the chloroplast envelope. The phosphatidate synthesized from a mixture of acyl-CoA or acyl–acyl-carrier protein species in chloroplast preparations was found to have oleate and palmitate in positions 1 and 2 respectively (Frentzen *et al.*, 1983). The acyl-selectivity properties of the plastid *sn*-glycerol 3-phosphate-acylating enzymes account, therefore, for the intramolecular distribution of acyl groups in certain chloroplast membrane lipids (Siebertz & Heinz, 1977; Siebertz *et al.*, 1980; Sparace & Mudd, 1982; Heinz & Roughan, 1983). The *sn*-glycerol 3-phosphate acyltransferase enzymes in higher plants consequently play a major role in determining the acyl composition of complex lipids, and in the present study were found to account fully for the non-random character of acyl-group distribution in the seed triacylglycerol (Gunstone & Ilyas-Qureshi, 1965; Gunstone *et al.*, 1965; Hilditch & Williams, 1964; Ichihara & Noda, 1980). In animal systems, the synthesis of phosphatidate involves two reactions, the first yielding a 1-monoacyl-*sn*-glycerol 3-phosphate (Yamashita & Numa, 1972; Monroy *et al.*, 1973; Tamai & Lands, 1974) and the second giving rise to the 1,2-diacyl-*sn*-glycerol 3-phosphate (Yamashita *et al.*, 1972; Ray *et al.*, 1970). The formation of a 1-monoacyl-*sn*-glycerol 3-phosphate from glycerol 3-phosphate and acyl-CoA in a particulate fraction from developing cotyledons of safflower has recently been demonstrated (Ichihara, 1984). The acyl specificity of the first acylation step as reported by Ichihara (1984) helps to confirm the results reported here on the acyl composition of position 1 of the *sn*-phosphatidate which accumulated in microsomal incubations with mixed [^{14}C]acyl-CoA substrates (Table 2). We have also found that microsomal preparations incubated with [^{14}C]palmitoyl-CoA and *sn*-glycerol 3-phosphate accumulate large amounts of 1-monopalmitoyl-*sn*-glycerol 3-phosphate (about 7 nmol·min^{-1}·mg of protein^{-1}) (S. Stymne, G. Griffiths & A. K. Stobart, unpublished work). Only traces (< 4%) of this lysophosphatidate, however, partition in the chloroform phase after the methanol/chloroform extraction. To achieve quantitative recovery of the lysophosphatidate it is necessary to extract the reaction mixtures with butanol/acetic acid as described in the Materials and methods section.

The enzymes involved in the acylation of glycerol 3-phosphate are accordingly of prime importance in governing the quality and quantity of the acyl components of the triacylglycerols in

developing oilseeds. The present work is concerned with the synthesis of triacylglycerol in the safflower variety Gila. Gila seed oil is particularly rich in linoleate, where it accounts for 70% of the fatty acids present (Stymne *et al.*, 1983). The activity of the 2-oleoyl-*sn*-phosphatidylcholine desaturase in microsomal membranes from this variety is extremely high (Stymne *et al.*, 1983; Stobart *et al.*, 1983) and hence the efficient production of linoleate coupled to acyl exchange between acyl-CoA and position 2 of *sn*-phosphatidylcholine would result in a linoleate-rich acyl-CoA pool. The channelling of oleate to phosphatidylcholine through acyl exchange appears to leave little oleoyl-CoA for the acylation of *sn*-glycerol 3-phosphate, and this is reflected in the relatively low content of oleate in the endogenous microsomal phosphatidate (Table 8). In other species and varieties, however, in which the production of C_{18} polyunsaturated fatty acids is less efficient, the oleate probably has more opportunity to participate in the acylation of position 1 of *sn*-glycerol 3-phosphate. In these situations the equilibration that occurs between diacylglycerol and phosphatidylcholine during the synthesis of triacylglycerol (Stymne & Stobart, 1984*a*; Stobart & Stymne, 1985) will assume greater importance in increasing the content of polyunsaturated fatty acids in the final oil. It is also noteworthy that the incorporation of radioactive palmitate or stearate into phosphatidylcholine (Table 3) is almost equivalent to the mass of the non-radioactive linoleate that is utilized in the synthesis of phosphatidate (Table 4). This is due to the acyl exchange between the saturated acyl-CoA, when provided as a single substrate, and the predominant fatty acid in position 2 of *sn*-phosphatidylcholine, i.e. linoleate. Non-radioactive linoleoyl-CoA is therefore made available through acyl exchange for the efficient acylation of position 2 of *sn*-glycerol 3-phosphate. The flow of linoleate from phosphatidylcholine to phosphatidate via acyl exchange is also clearly demonstrated in the pulse–chase experiment reported in Table 6. The results confirm previous observations, based on specific radioactivity data, on the movement of oleate to phosphatidylcholine and the return of linoleate to the acyl-CoA pool for the acylation of glycerol 3-phosphate (Stobart *et al.*, 1983). The present experiments were carried out with incubation mixtures containing EDTA and so the movement of radioactivity from phosphatidate to diacyl- and triacyl-glycerol was not monitored. In other work, however, we have clearly shown the utilization of phosphatidate in diacyl- and triacylglycerol synthesis (Stymne *et al.*, 1983; Stymne & Stobart, 1984*a*; Stobart & Stymne, 1985). The observations reported here are particularly significant in that they provide further evidence for acyl exchange which does not rely on the direct analysis of acyl-CoA. Previous demonstrations of the exchange have been based upon the fatty acids in the acyl-CoA pool and position 2 of *sn*-phosphatidylcholine (Stymne *et al.*, 1983; Stobart *et al.*, 1983). This, of course, requires the adequate measurement of acyl-CoA, and we have presented methods which in practised hands are reliable. It should also be noted that the state of the seed material is critical for obtaining systems which are capable of good rates of acyl exchange and triacylglycerol synthesis in vitro. The cotyledons must be fresh and not harvested from stored frozen seed, and they should be at a developmental stage where oil deposition has only just commenced. The average size of both safflower and soya-bean cotyledons at this precise stage is usually 15 to 20mg fresh wt./cotyledon pair. The soya-bean cotyledons recently used by Macey & Stumpf (1984) are therefore, almost certainly, too far advanced towards maturity (0.2g fresh wt./cotyledon pair) for the preparation of microsomal membranes with adequate biosynthetic activity.

The present work adds further credence to our proposals for the regulation of the acyl quality of the triacylglycerols that are synthesized in developing oilseed cotyledons (Stymne *et al.*, 1983; Stymne & Stobart, 1984*a*; Stobart & Stymne, 1985) and so provides experimental models for plant-breeding and genetic-engineering programs concerned with vegetable-oil production.

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