The interconversion of diacylglycerol and phosphatidylcholine during triacylglycerol production in microsomal preparations of developing cotyledons of safflower (*Carthamus tinctorius* L.)

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Microsomal preparations from the developing cotyledons of safflower (*Carthamus tinctorius*) catalyse the acylation of *sn*-glycerol 3-phosphate in the presence of acyl-CoA. Under these conditions the radioactive glycerol in *sn*-glycerol 3-phosphate accumulates in phosphatidic acid, phosphatidylcholine, diacyl- and tri-acylglycerol. The incorporation of glycerol into phosphatidylcholine is via diacylglycerol and probably involves a cholinephosphotransferase. The results show that the glycerol moiety and the acyl components in phosphatidylcholine exchange with the diacylglycerol during the biosynthesis of diacylglycerol from phosphatidic acid. The continuous reversible transfer of diacylglycerol with phosphatidylcholine, which operates during active triacylglycerol synthesis, will control in part the polyunsaturated-fatty-acid quality of the final seed oil.

INTRODUCTION

The glycerol moiety of *sn*-glycerol 3-phosphate in the presence of acyl-CoA is incorporated into phosphatidylcholine in microsomal preparations from the developing cotyledons of safflower (Stobart & Stymne, 1985), sunflower (*Helianthus annuus*) (Stymne & Stobart, 1984a) and linseed (*Linum usitatissimum*) (Stymne & Stobart, 1985). The incorporation is via diacylglycerol (Stymne & Stobart, 1984a) and is probably catalysed by a CDP-choline:1,2-diacylglycerol cholinephosphotransferase (cholinephosphotransferase, EC 2.7.8.2) (Roughan & Slack, 1982; Slack *et al.*, 1985). The activity of the cholinephosphotransferase offers the opportunity to return to phosphatidylcholine, for further desaturation, the C₁₈-unsaturated fatty acids that are destined for triacylglycerol (Stobart & Stymne, 1985). This would necessitate the reversibility of the enzyme, and Slack and co-workers (Slack *et al.*, 1983) have interpreted data from incorporation studies *in vivo* with tissue slices of developing linseed cotyledons as indicating such reversibility. We have proposed that there is a rapid reversible exchange of diacylglycerol with phosphatidylcholine when glycerol with associated acyl groups moves from phosphatidic acid through the so-called 'Kennedy' pathway to triacylglycerol (Stobart & Stymne, 1985). This has been based on the observation that radioactive glycerol is incorporated into phosphatidylcholine with no, or very little, synthesis *de novo* of this particular phospholipid. However, in conventional pulse-chase experiments with microsomal preparations it has been difficult to show convincingly the movement of glycerol and/or acyl groups in phosphatidylcholine to diacyl- and triacyl-glycerol. This lack of success is probably due to the relatively large pool of endogenous phosphatidylcholine in the microsomal membranes, which effectively dilutes the specific radioactivity. We have, however, now

succeeded in demonstrating, in microsomal preparations of safflower, the rapid exchange of diacylglycerol with phosphatidylcholine by using a different approach, and it is these experiments which are presented here.

MATERIALS AND METHODS

Chemicals

1-¹⁴C-labelled fatty acids and L-*sn*-[U-¹⁴C]glycerol 3-phosphate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin, L-*sn*-glycerol 3-phosphate, phospholipase A₂ [from Indian-cobra (*Naja naja*) venom], CoASH, 1-palmitoyl-*sn*-lysophosphatidylcholine and various fatty acids were purchased from Sigma.

[1-¹⁴C]Oleoyl-CoA (octadec-9-enoyl-CoA; sp. radioactivity 2836 d.p.m./nmol) and unlabelled acyl-CoA species were synthesized as described by Sanchez *et al.* (1973). [glycerol-¹⁴C]Lysophosphatidylcholine (1-acyl-*sn*-[U-¹⁴C]glycerol-3-phosphocholine; sp. radioactivity 2870 d.p.m./nmol) was prepared from [glycerol-¹⁴C]phosphatidylcholine by hydrolysis with phospholipase A₂ (Stymne & Stobart, 1984a) and purified in the t.l.c. system for polar lipids (see below). The [glycerol-¹⁴C]phosphatidylcholine was obtained from microsomal membrane preparations of safflower cotyledons that had been incubated with *sn*-[U-¹⁴C]glycerol 3-phosphate and linoleoyl-CoA as described elsewhere (Stobart & Stymne, 1985).

Plant material

Safflower (*Carthamus tinctorius* L.) plants, var. Gila, were grown from seed in a 16 h photoperiod at 28 °C with a light intensity of 20000 lux and an 8 h night at 22 °C. The plants were hand-pollinated and the seeds were harvested 14–18 days after flowering. Microsomal

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membranes were prepared from the seed cotyledons as described previously (Stymne & Stobart, 1984a).

Enzyme assays

All assays were done at 30 °C with constant shaking. Incubation mixtures contained 10 mg of bovine serum albumin, substrates and cofactors dissolved in 0.1 M-phosphate buffer, pH 7.2, at the concentration stated in the Tables and the Figure, and the microsomal preparation adjusted to a final volume of 1 ml with 0.1 M-phosphate buffer.

Analytical procedures

Reactions were terminated by the addition of 0.15 M-acetic acid and the lipids were extracted in a modification of the medium described by Bligh & Dyer (1959). The lower chloroform phase, which contains the complex lipids and the free fatty acids, was removed and evaporated to dryness under N₂. The residue was dissolved in a small volume of chloroform and the polar and the 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 I₂ vapour, were removed from the plates and either assayed for radioactivity or methylated with methanolic HCl (Kates, 1964) for the analysis of the fatty acids. The fatty acid methyl esters were analysed by g.l.c. with a glass column (2 m × 2 mm) containing 10% BDS on Chromosorb W (HP, 80–100 mesh) and quantified by using methylheptadecanoic acid as an internal standard.

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 ¹⁴C. All radioactivity counts were corrected for background and quenching.

RESULTS

Recently we showed (Griffiths *et al.*, 1985) that EDTA inhibited the activity of the phosphatidate phosphohydrolase (phosphatidase; EC 3.1.3.4.) in safflower microsomes, and under these conditions the membranes accumulated large quantities of phosphatidic acid when incubated with *sn*-glycerol 3-phosphate and acyl-CoA. The inhibitory effect of EDTA was overcome with Mg²⁺, and the accumulated phosphatidic acid was utilized in the synthesis of diacyl- and triacyl-glycerol. These properties of the phosphatidase have been used to design experiments to investigate the equilibration of diacylglycerol with phosphatidylcholine during the operation of the Kennedy pathway. The rationale behind this approach was as follows. Large quantities of phosphatidic acid can be made to accumulate in microsomal preparations incubated with *sn*-glycerol 3-phosphate, acyl-CoA and EDTA. Radiolabelled phosphatidylcholine can then be efficiently synthesized in the membranes by the acylation of added lysophosphatidylcholine with [¹⁴C]oleoyl-CoA (Stymne & Stobart, 1984b). In those microsomes that contain a large pool of phosphatidic acid and [¹⁴C]phosphatidylcholine, the addition of Mg²⁺ will bring about a rapid depletion of the phosphatidic acid and the synthesis of diacylglycerol. If the reverse reaction of the cholinephosphotransferase is operating, then the diacylglycerol will become radioactive due to its

equilibration with the pool of [¹⁴C]phosphatidylcholine. The typical results for such an experiment are given below.

Microsomal membranes with an increased pool size of phosphatidic acid were prepared by incubating the microsomes (equivalent to 460 nmol of phosphatidylcholine) with 2000 nmol of *sn*-glycerol 3-phosphate and EDTA (10 mM). At 5 min intervals, for a period of 80 min, 160 nmol of oleoyl-CoA was added to the incubation mixture (total acyl-CoA added, 2560 nmol). The membranes were then diluted with phosphate buffer and centrifuged at 100 000 *g* for 60 min to remove residual acyl-CoA, EDTA and *sn*-glycerol 3-phosphate. In order to radiolabel the phosphatidylcholine, the microsomal pellet was resuspended in fresh buffer and portions (equivalent to 46 nmol of phosphatidylcholine) were incubated for 5 min with 30 nmol of 1-palmitoyl-*sn*-lysophosphatidylcholine and 20 nmol of [¹⁴C]oleoyl-CoA in the absence of Mg²⁺. Mg²⁺ (20 mM) was then added to the incubation mixture, and the mass of the fatty acids and the distribution of [¹⁴C]oleate in the complex lipids (Table 1 and Fig. 1) were determined at regular intervals. Control microsomal membranes, prepared by pretreatment with EDTA and *sn*-glycerol 3-phosphate and without acyl-CoA substrate, were also treated as described.

Microsomes that had been incubated with *sn*-glycerol 3-phosphate and oleoyl-CoA and thereafter radiolabelled with [oleoyl-¹⁴C]phosphatidylcholine (Table 1, 'Zero time*') contained 22 nmol of phosphatidic acid in each incubation. The control microsomes, which were radiolabelled in the same way, contained, however, only trace amounts of phosphatidic acid (2 nmol; Table 1, 'Zero time†'). On addition of Mg²⁺ to the incubation mixture the phosphatidic acid declined over 60% within 30 min, and concomitant with this was an increase in the diacylglycerol. In the absence of Mg²⁺, virtually no changes were observed in the quantity of diacylglycerol or phosphatidic acid (Table 1). The results show that the treatment with Mg²⁺ overcame the almost complete block in the utilization of phosphatidic acid that had occurred with pretreatment in EDTA. Within a short time after the

Table 1. Effect of Mg²⁺ on the synthesis of diacylglycerol from phosphatidic acid in EDTA-treated microsomal preparations of developing cotyledons of safflower

Microsomes that had been pretreated with EDTA, *sn*-glycerol 3-phosphate and oleoyl-CoA were incubated for 5 min with lysophosphatidylcholine and [¹⁴C]oleoyl-CoA ('Zero time*') before the addition of Mg²⁺ (20 mM). Control microsomal preparations ('Zero time†', '120 min† + Mg²⁺') were pretreated similarly but without acyl-CoA.

Treatment	Phosphatidic acid (nmol)	Diacylglycerol (nmol)
Zero time*	22	8
30 min + Mg ²⁺	8	20
35 min – Mg ²⁺	21	8
120 min + Mg ²⁺	7	23
125 min – Mg ²⁺	27	9
Zero time†	2	2
120 min† + Mg ²⁺	4	4

Fig. 1. Interconversion of the [^{14}C]oleate in *sn*-[2-oleoyl- ^{14}C]-phosphatidylcholine with diacylglycerol during the production of diacylglycerol from phosphatidic acid in microsomal preparations of developing cotyledons of safflower

Microsomal preparations were pretreated with EDTA, *sn*-glycerol 3-phosphate and oleoyl-CoA and then incubated with lysophosphatidylcholine and [^{14}C]oleoyl-CoA for 5 min before the addition of Mg^{2+} . Control microsomal preparations were pretreated similarly, but acyl-CoA was omitted. At intervals the radioactivity in the lipids was determined. The results given are for single measurements only. The experiment, however, was repeated with a different microsomal preparation and gave similar results. Symbols used: Control microsomes + Mg^{2+} : $\circ$, phosphatidylcholine; $\triangle$, diacylglycerol; $\square$, triacylglycerol. Microsomes + Mg^{2+} : $\bullet$, phosphatidylcholine; $\blacktriangle$, diacylglycerol; $\blacksquare$, triacylglycerol. Microsomes - Mg^{2+} : $\circ$, phosphatidylcholine; $\triangle$, diacylglycerol; $\square$, triacylglycerol.

application of Mg^{2+} a relatively large quantity of phosphatidic acid was converted into diacylglycerol.

The interconversion of diacylglycerol and phosphatidylcholine is demonstrated by the movement of the [^{14}C]oleate between these lipids during the flow of glycerol from phosphatidic acid (Fig. 1). At 5 min after the addition of lysophosphatidylcholine and [^{14}C]oleoyl-CoA to the microsomal preparation and just before the addition of Mg^{2+} , almost all the [^{14}C]oleate had been utilized, and over 95% of this was recovered in phosphatidylcholine, with only trace amounts in the other lipids (Fig. 1, 0 min). Upon the addition of Mg^{2+} the relative proportion of [^{14}C]oleate in phosphatidylcholine decreased from 95% to below 50% after 120 min. Concomitant with this was an increase in the [^{14}C]oleate in diacyl- and triacyl-glycerol of over 40%. There was only a slight decline in the [^{14}C]oleate in phosphatidyl-

choline in the microsomal membranes incubated for 120 min in the absence of Mg^{2+} . In the control microsomal treatments there was very little transfer of radioactivity from phosphatidylcholine to diacyl- and triacyl-glycerol, even in the presence of Mg^{2+} .

The above experiment demonstrated the rapid reversible exchange of the acyl component in phosphatidylcholine with the diacylglycerol produced from phosphatidic acid. It was, however, considered necessary to establish whether the exchange also included the glycerol backbone in phosphatidylcholine. Microsomal preparations were therefore incubated with [^{14}C]lysophosphatidylcholine and oleoyl-CoA to radio-label the phosphatidylcholine pool. After sufficient lysophosphatidylcholine had been converted into phosphatidylcholine, non-radioactive *sn*-glycerol 3-phosphate and linoleoyl-CoA were added to the incubation mixtures, and the distribution of the radioactivity in the complex lipids was determined at regular intervals (Table 2). After 15 min incubation with the [^{14}C]lysophosphatidylcholine and oleoyl-CoA, over 85% of the radioactivity was present in phosphatidylcholine. At 3 h after the addition of *sn*-glycerol 3-phosphate and linoleoyl-CoA to the incubation mixture the radioactivity in the phosphatidylcholine was less than 40%, and associated with this was a concomitant increase in the radioactivity recovered in the diacyl- and triacyl-glycerol. Some transfer of glycerol from phosphatidylcholine to diacyl- and triacyl-glycerol was also observed in the control microsomes that had been incubated with [^{14}C]lysophosphatidylcholine and oleoyl-CoA for 195 min.

To confirm that the microsomes were capable of good rates of triacylglycerol synthesis, via the Kennedy pathway, preparations were incubated with *sn*-[^{14}C]glycerol 3-phosphate and linoleoyl-CoA (Table 2). After 3 h incubation, some 15 and 60% of the radioactivity was recovered in phosphatidylcholine and triacylglycerol respectively.

DISCUSSION

The results show quite clearly that the glycerol and acyl components in phosphatidylcholine efficiently exchange with diacylglycerol when glycerol backbone flows from phosphatidic acid towards triacylglycerol. The reversible transfer of diacylglycerol with phosphatidylcholine is probably catalysed by a cholinephosphotransferase (Roughan & Slack, 1982). Recently, Slack *et al.* (1985) examined the activity of this enzyme in microsomal preparations from safflower cotyledons and showed that the back-reaction from phosphatidylcholine to diacylglycerol could occur at about 10% of the rate of the forward reaction. These observations were based, however, on microsomal preparations in which there was no movement of glycerol from phosphatidic acid to triacylglycerol. Our experiments show that, under conditions of active oil synthesis, the diacylglycerol-phosphatidylcholine reaction is reversible and therefore can account for the return of diacylglycerol species, which contain oleate, to phosphatidylcholine for desaturation and the concomitant availability of polyunsaturated diacylglycerol for triacylglycerol production. The reaction therefore probably involves no net loss of phosphatidylcholine and hence offers greater energy conservation in a system that has evolved for the efficient production of seed storage oil.

Table 2. Movement of the [^{14}C]glycerol in [$g\text{lycerol-}^{14}\text{C}$]phosphatidylcholine to diacyl- and triacyl-glycerol during the acylation of *sn*-glycerol 3-phosphate and triacylglycerol synthesis in microsomal preparations of developing cotyledons of safflower

Microsomes, equivalent to 39 nmol of phosphatidylcholine, were incubated with 8 nmol [$g\text{lycerol-}^{14}\text{C}$]lysophosphatidylcholine and 20 nmol of oleoyl-CoA for 15 min ('15 min*'). After 15 min, 400 nmol of *sn*-glycerol 3-phosphate and 200 nmol of linoleoyl-CoA were added. At regular intervals thereafter the distribution of radioactivity in the complex lipids was determined. A control incubation just containing [$g\text{lycerol-}^{14}\text{C}$]lysophosphatidylcholine and oleoyl-CoA was carried out for 195 min ('195 min†'). To ascertain the activity of the Kennedy pathway, microsomes (equivalent 39 nmol of phosphatidylcholine) were incubated with 400 nmol of [^{14}C]-*sn*-glycerol 3-phosphate and 200 nmol of linoleoyl-CoA for 3 h ('3 h†...'), after which the radioactivity in the complex lipids were determined. The data are given as mean values for two incubations in each case.

Incubation time (min) and sample	Distribution of the radioactivity in the complex lipids (%)					
	Lysophosphatidylcholine	Phosphatidylcholine	Phosphatidic acid		Triacylglycerol	Other chloroform-soluble lipids
			(nmol)	(%)		
15 min*	7	85	0.1		2	3
15 min + 1 h	3	61	1		4	6
15 min + 2 h	3	46	2		6	8
15 min + 3 h	4	38	3		7	8
195 min†	13	60	1		4	8
		(nmol)	(nmol)	(%)	(nmol)	(nmol)
		8.6	4.9	9	33.9	2.5
		16			61	5
3 h† + [^{14}C]Glycerol 3-phosphate and linoleoyl-CoA						

Previously we suggested (Stymne & Stobart, 1984a) that the cofactors for the cholinephosphotransferase (CMP, CDP-choline) do not appear to be rate-limiting and are probably present in catalytic amounts and tightly bound to the microsomal membranes. In this context it is noteworthy that we (A. K. Stobart & S. Stymne, unpublished work) have succeeded in incorporating [^{14}C]glycerol into phosphatidylcholine via diacylglycerol to an extent that represents some 50% of the microsomal phosphatidylcholine pool, with little change in mass. The thermodynamics of the interconversion catalysed by the cholinephosphotransferase in developing oilseeds appears to be rather novel and requires further work.

It is noteworthy that in the present study we observe some triacylglycerol synthesis from phosphatidylcholine in the apparent absence of acyl-CoA. It is possible that further reactions involving complex lipids can give rise to triacylglycerol. However, it is more probable that the acyl-CoA for the final acylation of diacylglycerol is made available by the back-reaction of the acyl-CoA:lysophosphatidylcholine acyltransferase that occurs in microsomal preparations from developing oilseeds (Stymne & Stobart, 1984b).

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