Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations from the developing seeds of *Cuphea lanceolata*

Maureen BAFOR,* Lisbeth JONSSON, Allan Keith STOBART† and Sten STYMNE‡

Department of Plant Physiology, Swedish University of Agricultural Sciences, P.O. Box 7047, S-750 07 Uppsala, Sweden

Embryos of *Cuphea lanceolata* have more than 80 mol % of decanoic acid ('capric acid') in their triacylglycerols, while this fatty acid is virtually absent in phosphatidylcholine (PtdCho). Seed development was complete 25–27 days after pollination, with rapid triacylglycerol deposition occurring between 9 and 24 days. PtdCho amounts increased until day 15 after pollination. Analysis of embryo lipids showed that the diacylglycerol (DAG) pool consisted of mainly long-chain molecular species, with a very small amount of mixed medium-chain/long-chain glycerols. Almost 100 % of the fatty acid at position *sn*-2 in triacylglycerols (TAG) was decanoic acid. When equimolar mixtures of [¹⁴C]decanoic and [¹⁴C]oleic acid were fed to whole detached embryos, over half of the radioactivity in the DAG resided in [¹⁴C]oleate, whereas [¹⁴C]decanoic acid accounted for 93 % of the label in the TAG. Microsomal preparations from developing embryos at the mid-stage of TAG accumulation catalysed the acylation of [¹⁴C]glycerol 3-phosphate with either decanoyl-CoA or oleoyl-CoA, resulting in the formation of phosphatidic acid (PtdOH), DAG and TAG. Very little [¹⁴C]glycerol entered PtdCho. In combined incubations, with an equimolar supply of [¹⁴C]oleoyl-CoA and [¹⁴C]decanoyl-CoA in the presence of glycerol 3-phosphate, the synthesized PtdCho species consisted to 95 % of didecanoic and dioleic species. The didecanoyl-glycerols were very selectively utilized over the dioleoylglycerols in the production of TAG. Substantial amounts of [¹⁴C]oleate, but not [¹⁴C]decanoate, entered PtdCho. The microsomal preparations of developing embryos were used to assess the acyl specificities of the acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15) and the acyl-CoA:*sn*-1-acylglycerol-3-phosphate acyltransferase (LPAAT, EC 2.3.1.51) in *Cuphea lanceolata* embryos. The efficiency of acyl-CoA utilization by the GPAT was in the order decanoyl > dodecanoyl > linoleoyl > myristoyl = oleoyl > palmitoyl. Decanoyl-CoA was the only acyl donor to be utilized to any extent by the LPAAT when *sn*-decanoylglycerol 3-phosphate was the acyl acceptor. *sn*-1-Acylglycerol 3-phosphates with acyl groups shorter than 16 carbon atoms did not serve as acyl acceptors for long-chain (≥ 16 carbon atoms) acyl-CoA species. On the basis of the results obtained, we propose a schematic model for triacylglycerol assembly and PtdCho synthesis in a tissue specialized in the synthesis of high amounts of medium-chain fatty acids.

INTRODUCTION

Few plant species are known to accumulate triacylglycerols (TAG) that are rich in the medium-chain fatty acids (< 16 carbon atoms). The major sources of such fatty acids are the economically important coconut and oil-palms. An annual plant species accumulating high levels of medium-chain fatty acids in the TAG of the seed is *Cuphea* (Wilson *et al.*, 1960). A feature of the family to which *Cuphea* belongs (Lythraceae) that makes it a particularly useful experimental system to study the biosynthesis of TAG with medium-chain fatty acids is that several *Cuphea* species have oils almost exclusively composed of one or two medium-chain fatty acids, and these can range from C₈ to C₁₄ (Graham *et al.*, 1981).

The biosynthesis of TAG in oilseeds involves at least two separate cell compartments. Primary acyl groups are synthesized by the fatty acid synthetase (FAS) enzyme complex localized in the plastid (Ohlrogge *et al.*, 1979), whereas the acylation of the glycerol backbone and the desaturation of oleic acid to C₁₈ polyunsaturated fatty acids occur in the endoplasmic reticulum (Stymne & Stobart, 1987). What regulatory factors determine the chain length of the fatty acid products of the FAS are unknown,

although studies *in vitro* suggest that the activity of the acetyl-CoA transacylase enzyme (Shimikata & Stumpf, 1983) and/or the concentration of acyl-carrier protein (Huang & Stumpf, 1971; Slabas *et al.*, 1982a; Singh *et al.*, 1984) may be involved.

The synthesis of C₁₈ polyunsaturated fatty acids from oleic acid and their incorporation into TAG in oilseeds are better understood (Stymne & Stobart, 1987). It has been shown for many species that diacylglycerols (DAG) are common precursors of phosphatidylcholine (PtdCho) and TAG. During the assembly of triacylglycerols, through the so-called 'Kennedy' (1961) pathway, there is an equilibration of glycerol backbone between the pools of DAG and PtdCho (Slack *et al.*, 1985; Stobart & Stymne, 1985). *Cuphea* seeds containing TAG with medium-chain fatty acids, however, have only trace amounts of these acids in PtdCho and in other membrane lipids (Slabas *et al.*, 1982b; Ohlrogge, 1988).

Here we report work on lipid synthesis in the developing seeds of *Cuphea lanceolata* (C₁₀ species), with particular emphasis on the mechanisms by which medium-chain fatty acids are excluded from membrane lipids, and particularly PtdCho, and dedicated towards the construction of TAG.

Abbreviations used: DAG, diacylglycerol(s); Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); Gro3P, *sn*-glycerol 3-phosphate; GPAT, acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase; lysoPtdOH, lysophosphatidic acid (*sn*-1-acylglycerol 3-phosphate); LPAAT, acyl-CoA:*sn*-1-acylglycerol-3-phosphate acyltransferase; PtdOH, phosphatidic acid; PtdCho, phosphatidylcholine; TAG, triacylglycerol(s); FAS, fatty acid synthetase.

* Permanent address: Biochemistry Division, Nigerian Institute for Oil Palm Research, P.M.B. 1030, Benin City, Nigeria.

† Permanent address: Department of Botany, University of Bristol, Bristol BS8 1UG, U.K.

‡ To whom correspondence and reprint requests should be sent.

MATERIALS AND METHODS

Chemicals

[1-¹⁴C]Decanoic acid was purchased from CEA, Gif-sur-Yvette, France. [1-¹⁴C]Oleic acid and [U-¹⁴C]*sn*-glycerol 3-phosphate [U-¹⁴C]Gro3P were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. BSA (fraction V; fatty-acid-free), CoASH, triacylglycerol lipase (from *Rhizopus arrhizus*), Gro3P [di(mono-hexylammonium) salt], *sn*-1-oleoyl-*sn*-glycerol 3-phosphate (oleoyl lysophosphatidic acid, oleoyl lysoPtdOH) and the various non-radioactive acids were purchased from Sigma Chemical Co.

[1-¹⁴C]Decanoyl-CoA and [1-¹⁴C]oleoyl-CoA (at the specific radioactivities stated in the Tables and Figures) and various non-radioactive acyl-CoA species were synthesized from their mixed anhydrides as described by Sánchez *et al.* (1973). Medium-chain PtdOH species were synthesized by allowing a mixed fatty acid trifluoroacetic anhydride to react with Gro3P essentially by the method of Kanda & Wells (1981) for PtdCho synthesis, but with the following modifications: *sn*-glycero-3-phosphocholine was replaced by Gro3P and all reagents and volumes were down-scaled to 1/20th. The reaction between the fatty acid trifluoroacetic anhydride and Gro3P was allowed to proceed for 8 min, after which 1 ml of methanol was added to the reaction mixture and the solution evaporated to near dryness under a stream of N₂ at room temperature. The residue was dissolved in 8 ml of 0.15 M-acetic acid and 3.75 ml of methanol/chloroform (2:1, v/v). After further addition of 1.25 ml of chloroform and mixing, a two-phase system was obtained; the lower (chloroform) phase (approx. 2 ml) was removed and applied on the silica-gel t.l.c. plates and chromatographed as described below for polar lipids. The lysophospholipids (*R_f* 0.16–0.18), which were revealed as white spots after spraying the plate with water, were eluted from the gel with 5 ml of methanol containing 1% acetic acid. To achieve complete isomerization of the *sn*-2-acyl-lysoPtdOH to the more stable *sn*-1-acyl-lysoPtdOH species the methanol solutions were kept at room temperature for 1 week before evaporation of the solvent and dissolving the product in water to a concentration of 1 nmol/μl. The total yield was 5–10% when calculated on the amount of added Gro3P.

Plant material and microsomal preparation

Cuphea lanceolata plants were grown from seeds in a 16 h photoperiod at 22 °C and an 8 h night at 17 °C. Flowers were hand-pollinated and the seeds harvested at various stages of development (14–18 days after pollination for microsomal preparations). The embryos were removed from the seed coat and stored on ice before further manipulations. The embryos were ground in a mortar with two parts (v/w) of 0.1 M-potassium phosphate buffer, pH 7.2, containing 0.1% BSA and 0.33 M-sucrose. The homogenate was filtered through a double layer of Miracloth, diluted 10-fold with fresh grinding medium, and centrifuged at 20000 *g* for 10 min. The supernatant was filtered through Miracloth and centrifuged at 105000 *g* for 90 min. The resulting microsomal pellet was resuspended in 0.1 M-potassium phosphate buffer, pH 7.2, and used immediately.

Protein determination

Protein was measured with the BCA protein assay reagent (Pierce Chemical Co.) after treatment of the sample with 0.1% SDS, and with BSA as standard.

Assays

Experiments with whole detached embryos were performed at 30 °C with ¹⁴C-labelled substrates and buffers as described in the Tables. At the end of the incubations, the buffers were discarded.

The embryos were washed with 0.1 M-phosphate buffer to remove residual radioisotopes and then homogenized with an Ultra-Turrax instrument in the mixture described by Bligh & Dyer (1959).

Microsomal incubations with radioactive substrates were carried out with constant shaking and with acyl-CoA species and acyl acceptor substrates, cofactors and microsomal membranes (as given in the Tables and Figures) in a final volume of 1 ml with 0.1 M-phosphate buffer, pH 7.2. Reactions were terminated by the addition of 1 ml of 0.15 M-acetic acid, and the lipids were extracted by addition of 3.75 ml of methanol/chloroform (2:1, v/v) and 1.25 ml of chloroform as modified from the method of Bligh & Dyer (1959). Incubations of acyl-CoA and [¹⁴C]Gro3P contained at least 150 μg of microsomal protein when TAG accumulation was studied. At this and higher microsomal concentrations, TAG were the major radioactive product, whereas incubation with lower amounts of membranes gave PtdOH as the main labelled product.

sn-Glycerol-3-phosphate acyltransferase (GPAT) and the 1-acylglycerol 3-phosphate acyltransferase (lysophosphatidic acid acyltransferase; LPAAT) activities were measured spectrophotometrically at 25 °C by the coupling of the free CoA, liberated from acyl-CoA, with 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs₂) as described by Bafor *et al.* (1990) by methods modified from those of Lands & Hart (1965). Concentrations of microsomal membranes and substrates are given in the Tables.

Activities of the GPAT were measured during 3 min after a lag time of 60 s. The acylation rates usually progressively increased during the measurement, giving an average activity over the 3 min of about half the maximal velocity. Since the assay does not distinguish between the first (GPAT-catalysed) and the second (LPAAT-catalysed) acylation steps, the increasing rate may be due to acylation of lysoPtdOH, the product formed as a result of the GPAT activity. The results from measurements of the GPAT activity might therefore, in some cases be an over-estimation of the real activity.

LPAAT was measured during 1–3 min after a lag time of 30 s and was linear throughout the measurement.

The validity of the spectrophotometric measurements were checked by using radioactive acyl-CoA substrates in the incubation mixtures. At the end of the spectrophotometric measurements the reaction mixture was extracted and the radioactive PtdOH quantified as described below. Good agreements were obtained between the spectrophotometric and radioactivity measurements. The addition of Nbs₂ had no effect on the LPAAT activity.

Analytical procedure

Chloroform phases from the extracted incubation mixtures were evaporated to dryness under N₂. 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.) and hexane/diethyl ether/acetic acid (50:50:1, by vol.) respectively. Areas with unsaturated lipids were located by lightly staining with I₂ vapour, after which the plate was sprayed with water. The disaturated PtdOH species and disaturated DAG appeared as intense white spots. Dioleoyl, oleoyl/decanoyl and didecanoyl PtdOH species were clearly separated from each other (*R_f* 0.78, 0.70 and 0.63 respectively). Also, the unsaturated DAG were separated from the disaturated DAG (*R_f* 0.41 and 0.35 respectively). However, it was important that separation of the DAG was carried out immediately after extraction of tissue or microsomes to minimize isomerization of *sn*-1,2-DAG to *sn*-1,3-DAG. The *sn*-1,3 disaturated glycerols co-chromatographed with the unsaturated *sn*-1,2-DAG. Reference

didecanoyl PtdCho co-migrated with reference decanoyl lyso-PtdOH (R_f 0.17). However, no endogenous didecanoyl PtdCho was detected in lipid extracts from embryos and microsomal preparations. In chloroform/methanol/water extractions (Bligh & Dyer, 1959) the major part of the decanoyl-lysoPtdOH resided in the upper methanol/water phase.

Lipid areas were removed from the plates and either assayed for radioactivity or methylated *in situ* with methanolic HCl (Kates, 1964) for the analysis of the fatty acids. The unlabelled and radioactive fatty acid methyl esters were analysed by g.l.c. and radio-g.l.c. respectively.

Analyses of the positional distribution of the fatty acids (radioactive and non-radioactive) in DAG and TAG were performed after treatment with TAG lipase from *Rhizopus arrhizus*. The chromatographed DAG and TAG were eluted from the t.l.c. gel with chloroform/methanol (2:1, v/v) and the solvent removed in a stream of N_2 . The lipids were dissolved in 0.5 ml of diethyl ether, after which 1 ml of 0.1 M-Tris/HCl buffer, pH 7.7, containing 5 mM- $CaCl_2$ and 1200 units of *Rhizopus arrhizus* lipase was added. The mixture was vigorously mixed at room temperature on a Microid (Griffin and George, Wembley, Middx., U.K.) flask shaker for 3 h. After removal of the diethyl ether in a stream of N_2 , the lipids were extracted by a modification of the medium described by Bligh & Dyer (1959). The chloroform phase was chromatographed in the t.l.c. system described above for neutral lipids. Lipid areas corresponding to the *sn*-2-monoacylglycerols and the non-esterified fatty acids (derived from position *sn*-1 of the *sn*-DAG and mixtures of positions *sn*-1 and *sn*-3 of the *sn*-TAG) were removed and methylated for further analysis by g.l.c. or radio-g.l.c.

Lipid samples were assayed for radioactivity in PCS (Amersham/Searle)/xylene (2:1, v/v) scintillant in LKB 1214 liquid-scintillation counter with an efficiency of 94 % for ^{14}C . All values were corrected for background and quenching.

Reproducibility

Owing to difficulties in obtaining large numbers of embryos at the right stage of development at one time, some of the data presented for microsomal enzyme activities are from single measurements. These experiments were repeated on separate occasions, with different batches of plant material, and gave similar qualitative results. The specific activities of GPAT and LPAAT (expressed as nmol/min per mg of microsomal protein) varied between batches with a factor up to 3, but results were identical with regard to substrate specificity and the ratio between GPAT and LPAAT activities.

RESULTS

Lipid deposition and composition during seed development

At 9 days after pollination the embryos had reached a stage where lipid analysis was feasible (fresh weight about 0.4 mg/embryo), and some 18 days later the seeds reached maturity. TAG deposition in the embryo occurred at a linear rate from 12 to 24 days after pollination. The content of PtdCho, on the other hand, increased only during the first 15 days and then remained constant (Fig. 1a). The relative amount of decanoic acid in the TAG increased rapidly from 9 to 12 days (from 70 to 80 mol %), and then at a lower rate: from 80 to 85 mol % between 12 and 24 days (Fig. 1b). Consequently the relative contribution of the major long-chain fatty acids, palmitic, oleic and linoleic, in the triacylglycerols, decreased throughout the whole period of oil deposition, although the absolute amount increased (Fig. 1c).

The fatty acid constituents of the TAG, DAG and PtdCho in

Fig. 1. Lipid and fatty acid accumulation in developing embryos of *Cuphea lanceolata*

(a) TAG and PtdCho accumulation. (b) Relative content of decanoic acid ($C_{10:0}$) in TAG. (c) Accumulation of palmitic acid, oleic acid and linoleic acid in TAG. Symbols: ○, total TAG; ●, PtdCho; ▲, palmitic acid; □, oleic acid; ■, linoleic acid.

embryos at the mid-stage of TAG accumulation (14–18 days after pollination) are given in Table 1. Decanoic acid accounted for over 80 % of the fatty acids in the TAG, whereas the bulk of the DAG (64 %) contained C_{18} unsaturated fatty acids, with less than 10 % decanoic acid. The disaturated species of DAG had almost the same decanoic acid content as the TAG, but with greater amounts of palmitic and stearic acid. PtdCho contained only traces of decanoic acid, the major fatty acids being palmitate, oleate and linoleate. Didecanoyl species of PtdCho, which would separate from unsaturated species in the t.l.c. system used, were not detected.

A stereospecific analysis of the fatty acids in the disaturated species of the DAG and TAG was carried out (Table 2). In almost all molecules of the disaturated DAG and TAG, position *sn*-2 was esterified with decanoic acid. The majority of the other fatty acid species were, therefore, located at position *sn*-1 of the saturated DAG and *sn*-1 and/or *sn*-3 of the TAG. In the unsaturated DAG, however, the most common fatty acid at position *sn*-2 was linoleate, whereas some 60 % of the total decanoic acid, as well as the major part of the other saturated fatty acids, was at position *sn*-1.

Acylation of Gro3P in microsomal preparations from developing *Cuphea lanceolata* embryos

Microsomal preparations from the developing embryos of *Cuphea lanceolata* catalysed the acylation of *sn*-[^{14}C]glycerol 3-phosphate with decanoyl-CoA or oleoyl-CoA. Fig. 2 illustrates the kinetics of this [^{14}C]glycerol incorporation into lipid. In the presence of decanoyl-CoA, some 2–3 times more TAG was formed as compared with incubations with oleoyl-CoA. DAG accumulated in the presence of oleoyl-CoA, but was hardly

Table 1. Mass and fatty acid composition of various lipids isolated from *Cuphea lanceolata* embryos at mid-stage of development (14–18 days after pollination)

Lipid	Amount (nmol/100 embryos)	Fatty acid distribution (mol %)							
		C _{10:0}	C _{12:0}	C _{14:0}	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}
TAG	33200	83.3	3.1	3.2	3.8	0.7	2.9	2.2	0.1
Di-saturated DAG	95	78.8	3.9	4.9	10.3	4.0	0	0.2	0
Unsaturated DAG	168	9.8	1.1	2.6	18.9	3.5	19.3	44.2	1.5
PtdCho	584	0.3	0.4	2.0	24.9	3.1	30.9	37.2	1.1

Table 2. Positional distribution of fatty acid in endogenous DAG and TAG from *Cuphea lanceolata* embryos at mid-stage of development (14–18 days after pollination)

Lipids isolated from embryos were treated with TAG lipase and the resulting *sn*-2-monoacylglycerols and non-esterified fatty acids were separated, methylated, then analysed by g.l.c. For experimental details, see the Materials and methods section.

Lipid	<i>sn</i> Position	Fatty acid distribution (mol %)							
		C _{10:0}	C _{12:0}	C _{14:0}	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}
TAG	1+3*	76.2	4.0	4.3	5.2	0.8	5.5	3.9	0.1
	2	97.3	0.2	—	0.3	0.1	1.4	0.6	0.1
Disaturated DAG	1	76.5	4.4	5.1	11.2	2.8	—	—	—
	2	96.6	0.5	0.4	1.9	0.6	—	—	—
Unsaturated DAG	1	26.0	1.7	4.6	26.1	3.9	14.4	23.0	0.3
	2	9.9	0.2	0.4	3.1	1.7	18.8	64.6	1.3

* Deduced from total fatty acid profile and *sn*-2 position of the triacylglycerol

Fig. 2. Time course of incorporation of [¹⁴C]Gro into lipids in incubations of microsomal preparations from developing *Cuphea lanceolata* embryos with decanoyl-CoA (a) or oleoyl-CoA (b) in the presence of *sn*-[¹⁴C]Gro3P

Microsomal preparations (equivalent to 0.65 mg of microsomal protein) were incubated with 200 nmol of either decanoyl-CoA (a) or oleoyl-CoA (b) in the presence of *sn*-[¹⁴C]Gro3P (2 μmol, sp. radioactivity 2000 d.p.m./nmol), BSA (10 mg) and MgCl₂ (10 μmol) in 0.1 M-phosphate buffer, pH 7.2, in a total volume of 1 ml at 30 °C for various periods of time. For experimental details of lipid analysis, see the Materials and methods section. Symbols: ○, PtdOH; ●, PtdCho; △, DAG; ▲, TAG.

detected in incubations with decanoate. Very little [¹⁴C]glycerol was found in PtdCho with either acyl substrate.

To study the acyl-selectivity properties of the acylating enzymes, time-course experiments were carried out, using microsomal membranes incubated with Gro3P and an equimolar mixture of [¹⁴C]decanoyl-CoA and [¹⁴C]oleoyl-CoA. Both acyl species were readily utilized in the synthesis of PtdOH and TAG (Fig. 3). After 20 min incubation, 95 % of the radioactivity in PtdOH was present as the didecanoyl and dioleoyl molecular species, and only 5 % was found in the mixed decanoyl/oleoyl population (Fig. 3a). The DAG contained almost exclusively [¹⁴C]oleate. In the TAG, both [¹⁴C]decanoate and [¹⁴C]oleate (at a constant ratio of 4:6) accumulated up to 80 min of incubation. Some 60 % of the radioactivity at position *sn*-2 of the *sn*-TAG, at 80 and 160 min of incubation, was in decanoic acid (results not shown). No [¹⁴C]decanoate was found in PtdCho, although the incorporation of [¹⁴C]oleate was substantial (Fig. 3a).

Utilization *in vivo* of exogenously supplied ¹⁴C-labelled substrates in detached *Cuphea* cotyledons

Equimolar mixtures of [¹⁴C]decanoic acid and [¹⁴C]oleic acid (25 nmol of the ammonium salt of each acid) were fed for 60 min to detached cotyledons obtained at a mid-stage in development (Table 3). Some 44 % of the added ¹⁴C-labelled fatty acids was incorporated into complex lipids, and nearly 70 % of this was in the TAG. Little of the label in the TAG resided in [¹⁴C]oleate (< 7 %), and none of this was present at position *sn*-2 (results not shown). A high proportion of [¹⁴C]oleate, however, was found in the total DAG (54 % of the radioactivity in that lipid). [¹⁴C]Decanoate was present in small amounts in the unsaturated DAG species (only 8.6 % of ¹⁴C in total DAG).

Fig. 3. Time course of incorporation of [^{14}C]decanoyl-CoA and [^{14}C]oleoyl-CoA into lipids in incubations of microsomal preparations from developing *Cuphea lanceolata* embryos with Gro3P and equimolar mixtures of [^{14}C]decanoyl-CoA and [^{14}C]oleoyl-CoA

Microsomal preparations (equivalent to 0.65 mg of microsomal protein) were incubated with [^{14}C]decanoyl-CoA and [^{14}C]oleoyl-CoA (100 nmol each; sp. radioactivity 3300 d.p.m./nmol) in the presence of Gro3P (2 μmol), BSA (10 mg) and MgCl_2 (10 μmol) in 0.1 M-phosphate buffer, pH 7.2, in a total volume of 1 ml at 30 $^\circ\text{C}$ for various periods of time. For experimental details of lipid analysis, see the Materials and methods section. Symbols: $\circ$, didecanoyl PtdOH; $\bullet$, dioleoyl PtdOH; $\triangle$, oleoyl/decanoyl PtdOH; $\blacksquare$, oleoyl PtdCho; $\square$, decanoyl PtdCho; $\blacklozenge$, oleoyl DAG; $\diamond$, decanoyl DAG; $\blacktriangle$, oleoyl TAG; $+$, decanoyl TAG.

Table 3. Incorporation [^{14}C]decanoate and [^{14}C]oleate into lipids after incubation of detached developing cotyledons of *Cuphea lanceolata* with equimolar mixtures of exogenous [^{14}C]decanoate and [^{14}C]oleic acid

Cotyledons (eight pairs/incubation) at mid-stage of development (14–18 days after pollination) were incubated with equimolar mixtures of ammonium salts of [^{14}C]decanoate and [^{14}C]oleic acid (25 nmol each, sp. radioactivity 1.15×10^5 d.p.m./nmol) in 0.5 ml of 0.1 M-phosphate buffer, pH 7.2, at 30 $^\circ\text{C}$ for 60 min. For analytical procedures see the Materials and methods section. Results are the means $\pm$ S.E.M. for two incubations.

Lipid	^{14}C -labelled fatty acid incorporated (nmol)
DAG species	
Disaturated	
[^{14}C]C $_{10:0}$	1.03 ± 0.06
Unsaturated	
[^{14}C]C $_{10:0}$	0.24 ± 0.06
[^{14}C]C $_{18:1}$	1.51 ± 0.10
TAG	
[^{14}C]C $_{10:0}$	$14.0 \pm 0.2^*$
[^{14}C]C $_{18:1}$	0.99 ± 0.02
Total complex lipids	22.1 ± 0.1

* Radio-g.l.c. analysis of lipase-treated TAG revealed only [^{14}C]C $_{10:0}$ in position *sn*-2.

Table 4. Incorporation of [^{14}C]glycerol, [^{14}C]decanoate and [^{14}C]oleate into lipids after incubation of detached developing cotyledons of *Cuphea lanceolata* with exogenous substrates

Cotyledons (ten pairs/incubation) at mid-stage of development (14–18 days after pollination) were incubated with [^{14}C]glycerol (25 nmol; sp. radioactivity 3.8×10^5 d.p.m./nmol) in the absence or presence of ammonium salts of either decanoic or oleic acid (400 nmol) in 0.2 ml of 0.1 M-phosphate buffer, pH 7.2, at 30 $^\circ\text{C}$ for 60 min. Results are the means $\pm$ S.E.M. for two incubations. Similar incubations but with radioactively labelled acyl substrate (sp. radioactivity 500 d.p.m./nmol) and non-labelled glycerol were also carried out. For analytical procedures, see the Materials and methods section.

Substrate	[^{14}C]Glycerol incorporated (pmol)		
	PtdCho	DAG	TAG
[^{14}C]Glycerol	38 ± 5	357 ± 6	1217 ± 65
[^{14}C]Glycerol + decanoic acid	51 ± 10	429 ± 23	2579 ± 177
[^{14}C]Glycerol + oleic acid	105 ± 23	1110 ± 19	1438 ± 2

Substrate	[^{14}C]Acyl groups incorporated (nmol)			
	PtdCho	DAG	TAG	Total complex lipids
[^{14}C]Decanoic acid + glycerol	0.45	6.9	339	351
[^{14}C]Oleic acid + glycerol	18.7	25.3	15.8	71.3

The effect of addition of decanoic acid or oleic acid on the ability of the cotyledons to incorporate exogenously supplied [^{14}C]glycerol into lipids was investigated (Table 4). Together with added decanoic acid, [^{14}C]glycerol incorporation into TAG was twice as high as compared with glycerol alone or in the presence of oleate, whereas the incorporation into DAG and PtdCho only showed slight increase. Addition of oleic acid gave the opposite changes: incorporation into TAG was similar to that with [^{14}C]glycerol alone, but the amount of radioactivity in the DAG and PtdCho increased some 2–3-fold as compared with the other treatments.

In another set of experiments, [^{14}C]decanoic acid or [^{14}C]oleic acid was supplied in the presence of unlabelled glycerol (Table 4). With [^{14}C]decanoic acid only 0.1 and 2.0 % of the radioactivity incorporated into the complex lipids was in PtdCho and DAG respectively. The TAG contained over 96 % of the label, an amount equivalent to a total of 340 nmol of decanoate (i.e. 85 % of the [^{14}C]decanoate provided). When [^{14}C]oleate was used, the major radioactive lipids were PtdCho and DAG (26 and 35 % of total label respectively). Only 22 % of the incorporated [^{14}C]oleate was in the TAG.

Acyl specificity of glycerol acylation in microsomal preparations from developing cotyledons

The acyl specificity of GPAT and LPAAT in microsomal preparations was determined by using a spectrophotometric assay (Table 5). The efficiency of acyl utilization by the GPAT was in the order: decanoate = dodecanoate > linoleate > myristate = oleate > palmitate. The LPAAT, measured with 1-oleoyl-lysoPtdOH as acyl acceptor, exhibited a more pronounced specificity than the GPAT, and was exceptionally active with decanoate. Oleate, linoleate and dodecanoate were utilized at some 30 % of the rate with decanoate. Myristate, palmitate and

Table 5. GPAT and LPAAT activities as a function of acyl-CoA species in microsomal preparations from developing *Cuphea lanceolata* cotyledons

Enzymic measurements were carried out spectrophotometrically in 1 ml incubation mixtures with 7.5 nmol of acyl-CoA. For the GPAT assays, each incubation contained 65 µg of microsomal protein and 2 µmol of Gro3P. LPAAT was measured with 14 µg of microsomal protein and 20 nmol of oleoyl lysoPtdOH. GPAT and LPAAT were measured with two separate microsomal batches. For experimental details, see the Materials and methods section. Results are means $\pm$ S.E.M. for two assays.

Acyl-CoA substrate	Enzymic activity (nmol/min per mg of protein)	
	GPAT	LPAAT
C _{10:0} -CoA	5.6 $\pm$ 0.2	126 $\pm$ 0
C _{12:0} -CoA	5.5 $\pm$ 0.3	34.4 $\pm$ 0.6
C _{14:0} -CoA	2.6 $\pm$ 0.3	12.1 $\pm$ 0.4
C _{16:0} -CoA	1.7 $\pm$ 0.2	15.1 $\pm$ 4.3
C _{18:0} -CoA	0.5 $\pm$ 0.2	10.1 $\pm$ 0.6
C _{18:1} -CoA	2.4 $\pm$ 0.2	41.3 $\pm$ 2.9
C _{18:2} -CoA	3.7 $\pm$ 0.0	39.3 $\pm$ 1.4

stearate, however, were the least efficient substrates and gave only 10 % of the activity observed with decanoate.

Table 6 shows the activity of the LPAAT with different combinations of acyl-CoA substrates and lysoPtdOH acyl

acceptors. With the decanoate acceptor, decanoyl-CoA was the only acyl donor used to any great extent, whereas myristoyl- and oleoyl-CoA were almost inactive. Similar acyl specificities were observed with the dodecanoyl and myristoyl acceptors, except that the dodecanoyl-CoA was utilized somewhat better and at 35–40 % of the rate with decanoyl-CoA. *sn*-Palmitoyl lysoPtdOH was a poorer acyl acceptor for decanoyl-CoA than the medium-chain acyl acceptors, but here the oleoyl-CoA served as an acyl donor. *sn*-Oleoyl lysoPtdOH was the most efficient acyl acceptor used for all the acyl-CoA species tested (see also Table 5).

The inclusion of BSA in the reaction mixtures generally had a stimulating effect on the activity of both the GPAT and LPAAT. The degree of stimulation depended on several factors, such as the concentration of acyl-CoA and microsomes, and this was not further investigated.

To study the selectivity of the LPAAT, microsomal membranes were incubated with equimolar [¹⁴C]decanoyl-CoA and [¹⁴C]oleoyl-CoA, at different ratios of decanoyl and oleoyl lysoPtdOH species. Conditions were such that only some 10 % of the acyl-CoA and 5 % of the acyl acceptor would be consumed by the end of the incubation period. Table 7 shows the molecular species of PtdOH synthesized. With equimolar acyl acceptor, the PtdOH species consisted of 9 % didecanoate, 32 % mixed decanoate/oleate and 60 % dioleate. An increase in the ratio of decanoyl to oleoyl lysoPtdOH species brought about an increase in the relative content of the didecanoate species of PtdOH and with a corresponding decrease in the mixed species. It should be noted that the radioactivity in the mixed species of PtdOH always resided exclusively in the [¹⁴C]decanoyl acyl chain in this

Table 6. LPAAT activity as a function of acyl-donor and acyl-acceptor quality in microsomal preparations from developing *Cuphea lanceolata* cotyledons

Assays were carried out spectrophotometrically with 110 µg of microsomal protein, 7.5 nmol of acyl-CoA and 20 nmol of 1-acyl lysoPtdOH. For experimental details, see the Materials and methods section. The results shown are from a representative experiment.

Acyl acceptor	Acyl-donor ...	Enzymic activity (nmol/min per mg of protein)			
		C _{10:0} -CoA	C _{12:0} -CoA	C _{14:0} -CoA	C _{18:1} -CoA
C _{10:0} lysoPtdOH		20.2	1.7	0.1	0.3
C _{12:0} lysoPtdOH		17.8	6.3	0.0	0.0
C _{14:0} lysoPtdOH		18.4	7.2	0.8	0.0
C _{16:0} lysoPtdOH		6.9	3.7	0.6	3.6
C _{18:1} lysoPtdOH		43.2	14.7	4.4	15.5

Table 7. Molecular species of PtdOH synthesized from equimolar amounts of [¹⁴C]decanoyl CoA and [¹⁴C]oleoyl-CoA in the presence of mixtures of *sn*-1-decanoyl (C_{10:0}) lysoPtdOH and *sn*-1-oleoyl (C_{18:1}) lysoPtdOH in incubations of microsomal preparations of developing cotyledons of *Cuphea lanceolata*

Microsomal preparations (36 µg of protein) were incubated with an equimolar mixture of [¹⁴C]C_{10:0}-CoA and [¹⁴C]C_{18:1}-CoA (7.5 nmol each; sp. radioactivity 3336 d.p.m./nmol) in the presence of mixtures (at ratios given in the Table) of *sn*-1-C_{10:0} lysoPtdOH and *sn*-1-C_{18:1} lysoPtdOH (total 40 nmol) and 160 µg of BSA in 0.1 M-phosphate buffer, pH 7.2, in a total volume of 1 ml for 4 min at 20 °C. For experimental details, see the Materials and methods section. The percentage data are the amounts of each molecular species relative to the total PtdOH species. Results are the means $\pm$ S.E.M. for two incubations.

C _{10:0} /C _{18:1} lysoPtdOH ratio	¹⁴ C]PtdOH species synthesized ...	C _{10:0} /C _{10:0}		C _{18:1} /C _{10:0} *		C _{18:1} /C _{18:1}	
		(nmol)	(%)	(nmol)	(%)	(nmol)	(%)
1		0.19 $\pm$ 0.01	8.7	0.68 $\pm$ 0.11	31.2	1.31 $\pm$ 0.04	60.1
3		0.16 $\pm$ 0.00	11.2	0.40 $\pm$ 0.01	28.0	0.87 $\pm$ 0.03	60.8
7		0.23 $\pm$ 0.01	19.3	0.28 $\pm$ 0.05	23.5	0.68 $\pm$ 0.01	57.1

* [¹⁴C]Decanoate was the only radioactive acyl group in the mixed species of PtdOH.

and in similar incubations with mixtures of decanoyl and oleoyl lysoPtdOH species and [^{14}C]decanoyl-CoA and [^{14}C]oleoyl-CoA.

DISCUSSION

It is evident from the results presented that, in embryos of developing seeds of *Cuphea lanceolata*, mainly two DAG molecular species are synthesized: didecanoic acid and di-long-chain DAG. Firstly, the analysis of the endogenous lipids showed that in the pool of unsaturated DAG, containing long-chain fatty acids, the relative amount of decanoic acid was not more than 10–17%. Secondly, when equimolar amounts of [^{14}C]decanoate and [^{14}C]oleic acid were supplied to intact embryos, the amount of [^{14}C]decanoate in the unsaturated DAG was only about 17% of that of [^{14}C]oleate. Thirdly, when microsomes were incubated with equimolar amounts of [^{14}C]decanoyl-CoA and [^{14}C]dioleoyl-CoA in the presence of Gro3P, didecanoyl and dioleoyl PtdCho species prevailed over mixed oleoyl/decanoyl species of PtdCho.

These observations can, to some extent, be explained by the properties of the first two acylating enzymes of the Kennedy pathway, namely GPAT and LPAAT. In assays to determine substrate specificity, the GPAT exhibited the highest activity with decanoyl-CoA and dodecanoyl-CoA as acyl donors. The LPAAT only utilized medium-chain acyl-CoA species for acylation of medium-chain lysoPtdOH species, indicating a metabolic block in the formation of *sn*-1-medium-chain/*sn*-2-long-chain PtdOH species. The microsomes synthesized very little mixed oleoyl/decanoyl PtdOH from Gro3P and mixtures of decanoyl-CoA and oleoyl-CoA, but exogenous *sn*-1-oleoyl lysoPtdOH was efficiently acylated to yield *sn*-1-oleoyl/*sn*-2-decanoyl PtdOH. In the presence of both oleoyl and decanoyl lysoPtdOH species and with [^{14}C]decanoyl-CoA and [^{14}C]oleoyl-CoA, dioleoyl PtdOH species prevailed and the proportion and amounts of *sn*-1-oleoyl/*sn*-2-decanoyl PtdOH synthesized was reduced at low oleoyl lysoPtdOH concentrations. This indicates that the LPAAT selectively utilizes oleoyl-CoA to acylate oleoyl lysoPtdOH at low concentration of this acyl acceptor. *In vivo*, the high level of decanoic acid relative to oleic acid and linoleic acid, coupled with the specificity, and perhaps selectivity, of the GPAT for decanoyl-CoA would certainly benefit the production of decanoyl lysoPtdOH and, consequently, low amounts of mixed species of PtdOH. The GPAT and LPAAT in *Cuphea lanceolata* were thus both clearly more adapted to utilize medium-chain acyl-CoA species than the enzymes from oilseeds of species which accumulate long-chain fatty acids (Ichihara, 1984; Ichihara *et al.*, 1987; Bafor *et al.*, 1990). Likewise, Oo & Huang (1989) reported that LPAAT determined in microsomal preparations from palm (*Syagrus cocoides*) endosperm (a tissue rich in medium-chain fatty acids) catalysed synthesis of didodecanoyl PtdOH species, whereas corresponding maize (*Zea mays*) and rapeseed (*Brassica napus*) enzymes were inactive towards *sn*-1-dodecanoyl lysoPtdOH.

Several properties of the DAG acyltransferase in *Cuphea lanceolata* seeds can be deduced from the results. In microsomal incubations with Gro3P and [^{14}C]decanoyl-CoA, the enzyme channelled all available didecanoyl DAG into TAG, indicating a very high activity with this substrate. That the enzyme also has a pronounced selectivity for didecanoyl DAG over that of di-long-chain DAG is evident from the composition of the endogenous lipids. Unsaturated DAG were available in higher amounts than disaturated DAG and were obviously not as readily used for the synthesis of TAG, which contained more than 80% decanoic acid. Moreover, in microsomal incubations with Gro3P and equimolar mixtures of [^{14}C]decanoyl-CoA and [^{14}C]oleoyl-CoA, the accumulated DAG contained [^{14}C]oleate, but never [^{14}C]decanoate. In these incubations, [^{14}C]decanoate

was the major radioactive fatty acid at position *sn*-2 of the *sn*-TAG indicating that the majority of the DAG channelled to the TAG were decanoate-containing species. Since 60% of the radioactivity in TAG resided in oleate, position *sn*-3 of the *sn*-TAG must have been acylated almost exclusively from the pool of [^{14}C]oleoyl-CoA. Consequently, this acyl donor was favoured over decanoyl-CoA. In experiments *in vivo* where equimolar amounts of [^{14}C]decanoic acid and [^{14}C]oleic acid were supplied to intact embryos, very little [^{14}C]oleate compared with [^{14}C]decanoate entered TAG, and none of this was in position *sn*-2, indicating a complete block in utilizing long-chain DAG for TAG production. (In the experiment *in vivo*, only 7% of the label in TAG was in [^{14}C]oleic acid, but it should be noted that the uptake of [^{14}C]oleic acid by the embryos was only about 20% of the uptake of [^{14}C]decanoic acid.) Cao & Huang (1987), studying the DAG acyltransferase in microsomes from the developing seeds of *Cuphea carthagenensis*, found little selectivity between oleoyl-CoA and dodecanoyl-CoA when the enzyme was assayed with exogenously supplied dioleoylglycerol as the acyl acceptor and at normal temperatures.

Linolenic acid accumulated in TAG throughout seed development. Since this acid in oilseeds is synthesized in PtdOH

Scheme 1. Proposed pathway for the formation of TAG and PtdCho in developing seeds of *Cuphea lanceolata*

Only the utilization of decanoic acid and oleic acid is depicted. Linoleic acid, however, might follow a similar path to the oleic acid. Bold letters indicate main reactions. I: Plastids synthesize mainly decanoic acid, but also some oleic acid, and these are exported to the cytosol and activated to acyl-CoA species. II: GPAT in the endoplasmic reticulum catalyses the synthesis of mainly *sn*-1-decanoyl lysoPtdOH and some *sn*-1-oleoyl lysoPtdOH. III: LPAAT only utilizes decanoate for the acylation of *sn*-1-decanoyl lysoPtdOH. The enzyme is able to acylate *sn*-1-oleoyl lysoPtdOH with decanoate, but this is of minor significance at low *sn*-1-oleoyl lysoPtdOH concentrations. The *sn*-1-oleoyl lysoPtdOH is, therefore, almost exclusively acylated with oleate. IV: Didecanoyl and dioleoyl species of PtdOH, the two main molecular species synthesized, are converted into their corresponding DAG by the PtdOH phosphohydrolase. The didecanoylglycerols are selectively removed for the net TAG production, whereas dioleoyl DAG are utilized for the net synthesis of PtdCHO by the CDP-choline:DAG cholinephosphotransferase, an enzyme which probably can discriminate against decanoyl-containing species of DAG. Little PtdCho $\leftrightarrow$ DAG interconversion occurs during active TAG deposition. Linoleate in the TAG therefore arises from the acyl-CoA pool via acyl exchange between oleoyl-CoA and the linoleate at position *sn*-2 of the *sn*-PtdCho (its presumed site of synthesis). V: The DAG acyltransferase is highly selective for didecanoyl DAG and utilizes both medium- and long-chain acyl-CoA species.

(Stymne & Appelquist, 1978; Slack *et al.*, 1979), a flow of acyl groups through PtdOH to TAG should be taken into account. However, the incorporation of radiolabelled Gro3P into PtdCho in microsomes obtained from embryos at the mid-stage of seed development was very low. Some [14 C]glycerol entered PtdCho *in vivo*, and this incorporation was stimulated by the presence of oleic acid, but not decanoic acid, indicating a relatively poor DAG→PtdCho conversion, which utilizes only the long-chain DAG. It is reasonable to assume that the specificity (or selectivity) properties of the CDP-choline:1,2-DAG cholinephosphotransferase (EC 2.7.8.2) (Slack *et al.*, 1985) contribute to the exclusion of decanoic acid from PtdCho. Substantial [14 C]oleate, but not [14 C]decanoate, entered PtdCho *in vitro* and *in vivo*, and this was probably via acyl exchange between the fatty acids at position *sn*-2 of *sn*-PtdCho and acyl-CoA (Stymne & Glad, 1981).

Our findings are summarized in Scheme 1 in a proposed scheme for the biosynthesis of TAG and PtdCho in the developing embryos of *Cuphea lanceolata*.

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