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Biosynthesis of triacylglycerols by developing sunflower seed microsomes ☆

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Abstract

In order to elucidate the biochemical pathway involved in the formation of plant triacylglycerols rich in C_{18} polyunsaturated fatty acids, microsomes from developing sunflower cotyledons were incubated with [1-^{14}C]oleate or [1-^{14}C]linoleate. Total lipids were extracted and separated into lipid classes. The molecular species of phosphatidylcholine, diacylglycerols and triacylglycerols were analyzed. After [^{14}C]oleate feeding differences were found in the labelling of phosphatidylcholine and diacylglycerols labelled molecular species, whereas distribution of these molecules was the same when [^{14}C]linoleate was supplied. The role of phosphatidylcholine-diacylglycerol interconversion that may be catalysed by CDP choline:diacylglycerol phosphotransferase (EC 2.7.8.2) in generating triacylglycerols rich in linoleate is discussed. All the results obtained corroborate previous studies involving phosphatidylcholine in the biosynthesis of such triacylglycerols and allowed us to suggest reactional mechanisms for the formation of the different triacylglycerols molecular species in sunflower seeds.

Keywords: *Helianthus annuus*; Sunflower seed; Microsomes; Biosynthesis; Triacylglycerols; Phosphatidylcholine; Molecular species

1. Introduction

The biosynthesis of triacylglycerols (TAG) has been studied in oilseeds in vivo as well as in vitro (see reviews of Frentzen (1993) and Miquel and Browse (1994)). These studies reported that the Kennedy pathway (Kennedy, 1961) observed in animals tissues, where TAG assembly proceeds in three consecutive acylations of the glycerol backbone, was involved in the formation of TAG containing saturated and monounsaturated C_{16} and C_{18} acyl moities (Gurr, 1980, Stumpf, 1980). This route was also operative in the formation of TAG having unusual acyl moities (Mukherjee, 1983; Stymne, Bafor, Jonsson, Wiberg, & Stobart, 1990).

For the biosynthesis of TAG rich in C_{18} polyunsaturated fatty acids, another route where phosphatidylcholine (PC) is implicated, was proposed by some authors (Roughan & Slack, 1982; Stymne & Stobart, 1987; Triki, Ben Hamida, & Mazliak, 1997). Roughan and Slack, (1982) suggested that polyunsaturated diacylglycerols (DAG) for TAG synthesis were produced from PC by the choline phosphotransferase-catalysed back reaction:

$$\text{DAG} + \text{CDP chol.} \leftrightarrow \text{PC} + \text{CMP}$$

where CDP chol. is cytidine diphosphocholine and CMP is cytidine monophosphate.

Little or no selectivity towards molecular species of DAG has been reported for this enzyme in oilseeds (Slack, Roughan, Browse, & Gardiner, 1985). Stymne and Stobart, (1987) added an acyl-exchange between PC and the acyl-CoA pool, which is catalysed by the combined back and toward reaction of an acyl-CoA:lyso PC acyltransferase (EC 2.3.1.23). This exchange allows the transfer of oleate to PC for its subsequent desaturation and the removal of the linole-

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ate formed for TAG production. On the other hand, it has been clearly established that PC was the substrate for the oleate-desaturase system in plants (Slack, Roughan, & Browse, 1979; Stymne & Glad, 1981; Demandre, Trémolières, Justin, & Mazliak, 1986).

This paper describes the biosynthetic pathway of TAG in sunflower cotyledons, a material rich in linoleate ($C_{18:2}$). Labelling experiments were performed with [^{14}C]oleate ($C_{18:1}$) or [^{14}C]linoleate supplied to microsomal preparations. The incorporation of radioactive fatty acids into the molecular species of the different lipids synthesized during time-courses was investigated, researching eventual precursor–product relationship between molecules. PC and DAG, the metabolic intermediates, as well as the TAG final product were analysed, the results allowed us to elucidate the metabolic reactions operating for the main formed TAG.

2. Results

2.1. Labelling of lipid classes

A time-course of incorporation of [^{14}C]oleate into lipids was carried out for 90 min with microsomes from developing seeds. Fig. 1(A) indicates that the labelled fatty acid was rapidly incorporated into PC during the first 10 min, then a small decrease followed by stabilization were observed. DAG were also marked at a linear rate from zero time to 20 min, then decreased suddenly. The TAG radioactivity increased regularly during the time-course. Phosphatidic acid (PA) remained the least labelled lipid.

Replacing [^{14}C]oleate by [^{14}C]linoleate as a precursor, provided the following results (Fig. 1B): PC remained the major lipid synthesized, it decreased from 10 to 30 min of incubation while the radioactivity accumulated into the DAG. PA presented an increasing incorporation, on the other hand TAG biosynthesis was low.

During time-course experiments, we also stated that microsomes incorporated more labelled oleate than linoleate. In fact, for a nearly equal radioactivity supplied at the starting of the incubations, all the lipids synthesized were less labelled when [^{14}C]linoleate was used, except PA. On the other hand, the radioactivity of PC was about 3-fold more important when the [^{14}C]oleate was the marker.

2.2. Labelling of molecular species of lipid classes

The three principal lipid classes (PC, DAG and TAG) synthesized during labelling experiments were separated in their different molecular species using radio-HPLC, Table 1 indicates relative distribution of labelled molecular species. Three molecular species of

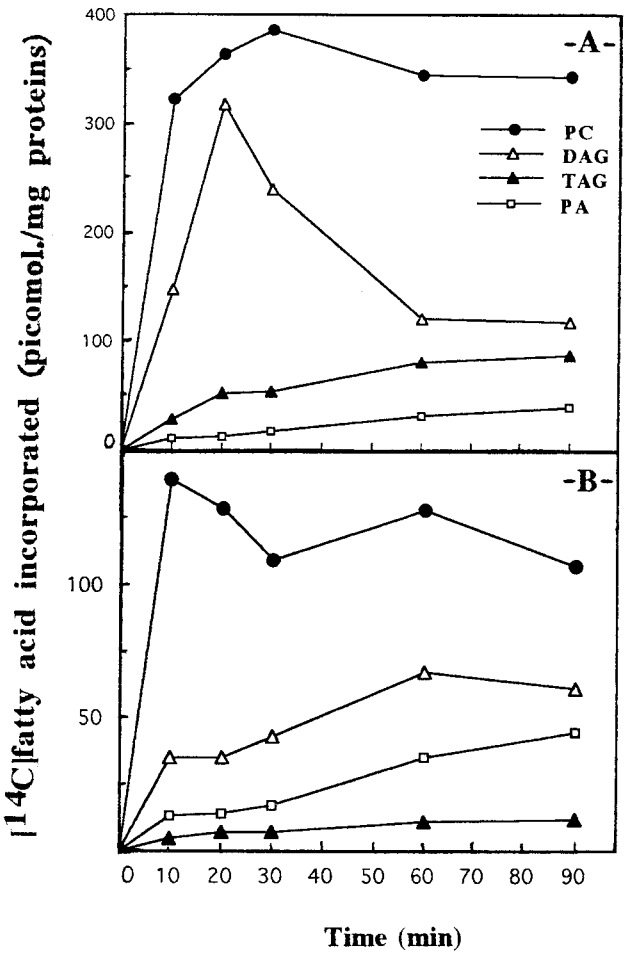


Fig. 1. Time-course of incorporation from [^{14}C]-oleate (**A**) or [^{14}C]linoleate (**B**) into lipid classes synthesized by sunflower seed microsomes. Batches of microsomes were supplied with labelled oleate or linoleate for 90 min. At each time lipids were extracted and separated, radioactivity in different lipids was determined.

PC were radioactive when [^{14}C]oleate was the substrate, the major one was 18:1/[^{14}C]18:1PC then a mixture of 18:2/[^{14}C]18:1PC with 16:0/[^{14}C]18:2PC as a minor part and 16:0/[^{14}C]18:1PC. The radiochromatograms of DAG-naphthylurethane derivatives presented small peaks on account of mass weakness of this lipid class and losses caused by derivatization. However, three labelled peaks were individualized; they correspond to the major molecular species 18:2/[^{14}C]18:1DAG which was eluted with 16:0/[^{14}C]18:2DAG as for PC, 18:2/[^{14}C]18:2DAG and a smaller third peak identified as 18:1/[^{14}C]18:1DAG. TAG analysis also showed three main labelled molecular species, the major part of them was constituted, at approximately the same ratio, by 18:1/18:1/[^{14}C]18:1TAG and 18:2/18:1/[^{14}C]18:1TAG mixed with 16:0/18:2/[^{14}C]18:1TAG as a minor part, then came 18:2/18:2/[^{14}C]18:1TAG. To confirm the [^{14}C]$C_{18:2}$ formation in our samples which were labelled with [^{14}C]$C_{18:1}$, we have analysed the three lipid classes fatty

Table 1
Relative distribution of the radioactivity in molecular species of lipid classes from microsomes supplied with [^{14}C]oleate or [^{14}C]linoleate after 90 min

Molecular species	PC		DAG		TAG	
	18:1[a]	18:2[b]	18:1[a]	18:2[b]	18:1[a]	18:2[b]
18:2/18:2[ab]	traces	32	33	40		
18:2[ab]/18:1[a] + 16:0/18:2[b]	39	68	54	60		
18:1/18:1[a]	51	–	13	–		
16:0/18:1°	10	–				
18:2/18:2/18:2[b]					–	8
18:2/18:2[b]/18:1[a]					13	50
18:2[b]/18:1/18:1[a] + 16:0/18:2[b]/18:1[a]					45	42
18:1/18:1/18:1[a]1[a]					42	–

[a] ^{14}C-oleate labelled.
[b] ^{14}C-linoleate labelled.

acids by argentation-TLC. Fig. 2 shows that DAG as well as PC contained labelled $C_{18:2}$, but TAG did not.

The relative proportions of the different molecular species separated from [^{14}C]linoleate labelled lipid class and calculated after 90 min of incubation are also presented in Table 1. Three molecular species of PC were synthesized, [^{14}C]18:2/18:1PC mixed with 16:0/[^{14}C]18:2PC as a minor proportion ([^{14}C]18:2/18:1PC remained the major molecular species during all the time course) and the third was 18:2/[^{14}C]18:2PC. On the other hand, we noticed a delay of incorporation into the DAG and the TAG, the first were labelled after only 60 min of incubation and the second after 90 min. Effectively these two lipid classes had a much lower acylation with labelled linoleate as observed above for Fig. 1, consequently quantitative determination of radioactivity was affected. The radiochromatogram of DAG naphthylurethanes showed two small radioactive peaks which corresponded to the same molecular species identified for PC, [^{14}C]18:2/18:1DAG mixed with 16:0/[^{14}C]18:2DAG and 18:2/[^{14}C]18:2DAG. We noticed that the 18:2/[^{14}C]18:2 species which appeared when the [^{14}C]oleate was used as substrate was then present among DAG as well as PC and at a nearly similar proportions. The principal molecular species of labelled TAG were: 18:2/[^{14}C]18:2/18:1TAG, [^{14}C]18:2/18:1/18:1TAG, but it was eluted with 16:0/[^{14}C]18:2/18:1TAG and 18:2/18:2/[^{14}C]18:2TAG which was weakly represented.

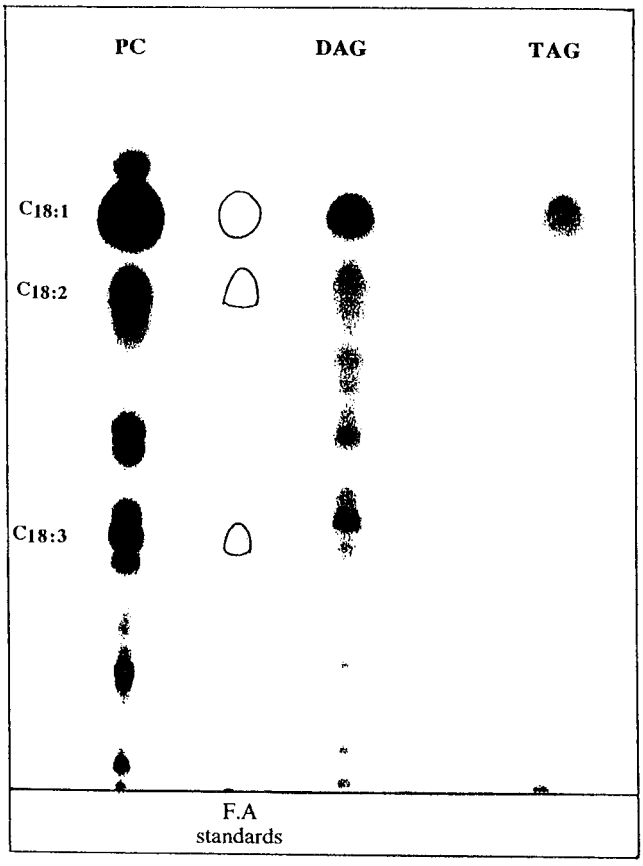


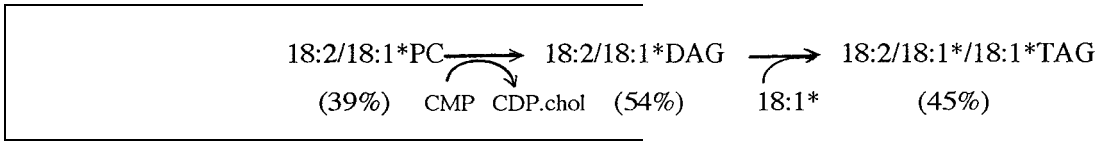
Fig. 2. Autoradiography of fatty acid methyl esters from [^{14}C]-oleate labelled lipid classes synthesized by microsomes and separated by argentation TLC.

3. Discussion

In this report the synthesis of TAG was investigated in the microsomal fraction prepared from developing cotyledons of sunflower. The main lipid classes involved in this process were analysed, PC appeared as the major one. The results obtained by radio-HPLC allowed us to suggest the reactional mechanisms by which the TAG were produced.

The time-course of [^{14}C]oleate acylation in the various lipid classes (Fig. 1A) evidenced a PC pool almost constant. It would be continuously recycled through the acyl exchange process occurring between PC (Stymne & Glad, 1981) and the acyl-CoA pool enriched with exogenous oleate and catalysed by the reversible lyso PC-acyltransferase (Stymne & Stobart, 1984b). The very high labelling of PC that we noticed when the precursor was the [^{14}C]oleate did confirm the oleate affinity of this enzyme in sunflower seeds (Stymne & Stobart, 1984a). The active acylation of lyso PC and the subsequent desaturation of the oleoyl PC would be coupled to the PC-DAG interconversion for which the back action of the choline phosphotransferase was involved (Slack, Campbell, Browse, & Roughan, 1983; Slack et al., 1985). The acylation of lyso PC with oleate seemed to be more rapid than the desaturation, which could explain the relative abundance of dioleoyl PC molecular species. In fact, radio-HPLC analysis showed a different molecular species

composition for PC and DAG Table 1. After 90 min of incubation, PC was richer in oleate (18:1/18:1PC, 51%), whereas DAG were richer in linoleate (18:2/18:1DAG mixed with 16:0/18:2DAG, 54%). Moreover, the 18:2/18:2 species reached 33% in DAG, while it was almost absent in PC. However, Fig. 2 indicated the presence of the [^{14}C]linoleate among PC fatty acids and also confirmed its presence in the DAG. This datum means that oleate desaturation occurred during the incubation and that the labelled18:2/18:1PC molecular species identified may be marked on its two acyl moeities. The [^{14}C]linoleate presence in the DAG could have two origins.

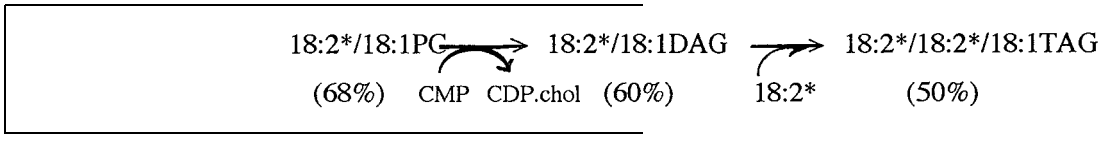


1. It appeared after the oleate-desaturation action, the enzyme was known to utilize oleoyl PC as a substrate (Demandre et al., 1986). This eucaryotic lipid biosynthesis pathway reaction is characteristic of plants and the main site of biosynthesis is the endoplasmic reticulum. The resulting linoleoyl PC should be transformed to linoleoyl DAG and the reverse choline phosphotransferase has been implicated as the mediator of this conversion. That the enzyme would have a strong selectivity for polyunsaturated PC molecular species in the back-reaction, explain the weak presence of 18:2/18:2PC in our samples and its relative abundance among DAG. Furthermore, DAG would accumulate as long as the TAG production was not very active. The PC linoleoyl moiety would also be released by acyl-exchange with the acyl-CoA pool catalysed by the reversible acyl-CoA:lyso PC-acyltranferase, then it could be utilized in the acylation of glycerol 3-phosphate to yield PA and DAG through the Kennedy pathway.
2. We could also hypothesize that the oleoyl DAG was a direct substrate for the oleate-desaturase: although, this eventuality has not been cited in the literature, it approaches the result found by Kesri and Mazliak (1995) where the microsomal linoleate-desaturase of carthamus cotyledons was able to utilize the linoleoyl DAG as a substrate. The rich linolenoyl DAG formed could be transferred to the chloroplasts for the biosynthesis of galactolipids. However, it is known that within the microsomes, the linoleoyl PC is the usual substrate of the ω^3 desaturase (Serghini-Caid, Demandre, Justin, & Mazliak, 1988).

The results obtained with the [^{14}C]oleate labelling experiments, using also the relative distribution of radioactivity in molecular species synthesized after 90 min incubation, allowed us to suggest the biosynthetic pathway of certain TAG molecular species. We supposed that during the incubation, acylations were carried out essentially with the marked fatty acid supplied.

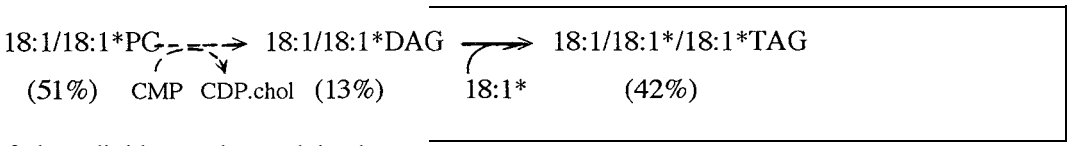
We noticed an extensive incorporation of the radioactivity in the 18:2/18:1DAG, which could result from the labelling of the 16:0/18:2 molecular specie eluted in the same time and perhaps the fact that TAG biosynthesis was less rapid than for DAG. Then this TAG molecular species could involve PC participation and the back-reaction of the choline-phosphotransferase.

When we supplied the [^{14}C]linoleate to the microsomal preparations, a PC-DAG transformation was evidenced throughout the 10–30 min period of the time-course (Fig. 1B), we observed a PC radioactivity decline and a DAG radioactivity increase. The reaction catalyzed by the phosphotransferase seemed to be activated when PC was richer in $C_{18:2}$. The slow rate of labelling of the TAG suggests a lesser affinity of DAG-acyltransferase for linoleate than for oleate. Results obtained by radio-HPLC analysis showed a great similarity between the PC and DAG molecular species distributions (Table 1); in the case where the linoleate was provided exogenously, the desaturation of oleate became redundant, then the two interconvertible lipid classes kept the same fatty acid compositions. The main TAG molecular species formed were [^{14}C]18:2/18:1/18:1TAG for which the biosynthetic pathway was discussed above and 18:2/[^{14}C]18:2/18:1TAG for which we suggest the following mechanism:



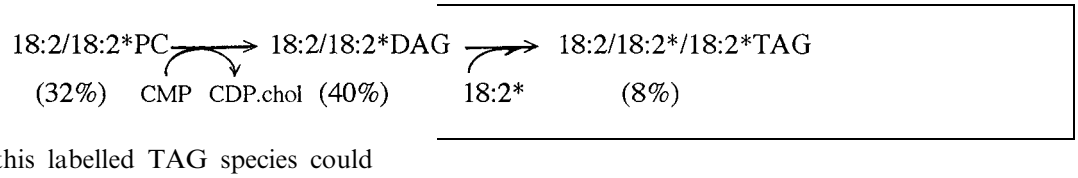
The two heterogenous TAG molecular species were labelled throughout all experiments using [^{14}C]oleate and [^{14}C]linoleate as precursors. The other molecular species were homogenous and could not be evidenced

except with one of the two markers: this was the case for triolein and for trilinolein. Their pathways could be established without ambiguity and were confirmed by the results. From [^{14}C]oleate labelling experiments, we could suggest these reactions, for the first:



The labelling ratios of these lipids can be explained as follows: the dioleoyl PC abundance will be generated from the important action of the lyso PC-acyltransferase which has a strong affinity for the oleoyl-CoA in sunflower seeds; the weak presence of the labelled diolein was justified if we take the affinity of the phosphotransferase in the back-reaction into account. It appeared to have no preference for monounsaturated molecular species of PC. Indeed, as shown by many studies, PC is involved in the production of TAG containing polyunsaturated fatty acids, where it acts as substrate for oleate-desaturase. Then, for triolein formation, the desaturation does not occur and the PC and phosphotransferase participations become useless. Labelled triolein obtained could be generated from unlabelled diolein after acylation with [^{14}C]oleate, through the classical reactions of the Kennedy pathway.

In contrast, trilinolein could be formed by the following pathway, involving PC and phosphotransferase action.



The small ratio of this labelled TAG species could indicate a lesser affinity of the DAG acyltransferase for the linoleate than for the oleate, or perhaps for the second substrate, the 18:2/18:2DAG.

Finally, two enzymes were particularly active in our plant material:the lyso PC-acyltransferase and the CDP choline:DAG-phosphotransferase, which were absent from the Kennedy pathway. On the other hand, two main heterogenous TAG molecular species were synthesized 18:2/18:1/18:1TAG and 18:2/18:2/18:1TAG; they needed the participation of PC for desaturation of oleate. It was also the case of the minor homogenous species, trilinolein; instead triolein could be synthesized by the Kennedy pathway classic reactions. The incubation of the microsomes were performed without addition of glycerol 3-phosphate, despite this fact a substantial part of the radioactivity was recovered in PA and TAG, an operative Kennedy pathway was then confirmed in our assays.

If [^{14}C]oleate was the labelled precursor, then PC and DAG had different distributions of molecular species, which became nearly similar when the [^{14}C]linoleate was utilized. These findings confirm the role of PC in oleate desaturation and suggest a selectivity for the reverse reaction of the phosphotransferase. It was possible that DAG species containing oleate were preferred in the transfer to PC, but PC species containing linoleate were preferred for the back reaction.

The synthesis of DAG from PC in the absence of CMP suggests that a substantial endogenous pool of CMP in our microsomal preparations may be available to the choline phosphotransferase backward reaction.

Experiments by feeding PC with labelled oleate to the microsomes have been done in our laboratory and effectively labelled DAG have been detected among the synthesized lipids 'in vitro' (Triki, Ben Hamida, & Mazliak, 1998). The following scheme (Fig. 3) summarizes the presumed molecular mechanism by which the microsomes from developing sunflower seeds synthesized TAG. Reactions derived from the well known Kennedy pathway and we added those necessary for PC turn-over providing linoleic acid, the characteristic fatty acid of sunflower oil.

4. Experimental

4.1. Chemicals

[1-^{14}C]labelled oleic and linoleic acids were provided from the CEA (France).

4.2. Plant material

Helianthus annuus L. seeds, var Forsol were obtained from CETIOM (Paris). Plants were grown in the university field (Tunis), between March and July. Maturing seeds were harvested 3 weeks after flowering and kept on ice.

4.3. Preparation of microsomes

All further procedures were carried out at −4°C, according to Kader (1977). The cotyledons (100 g)

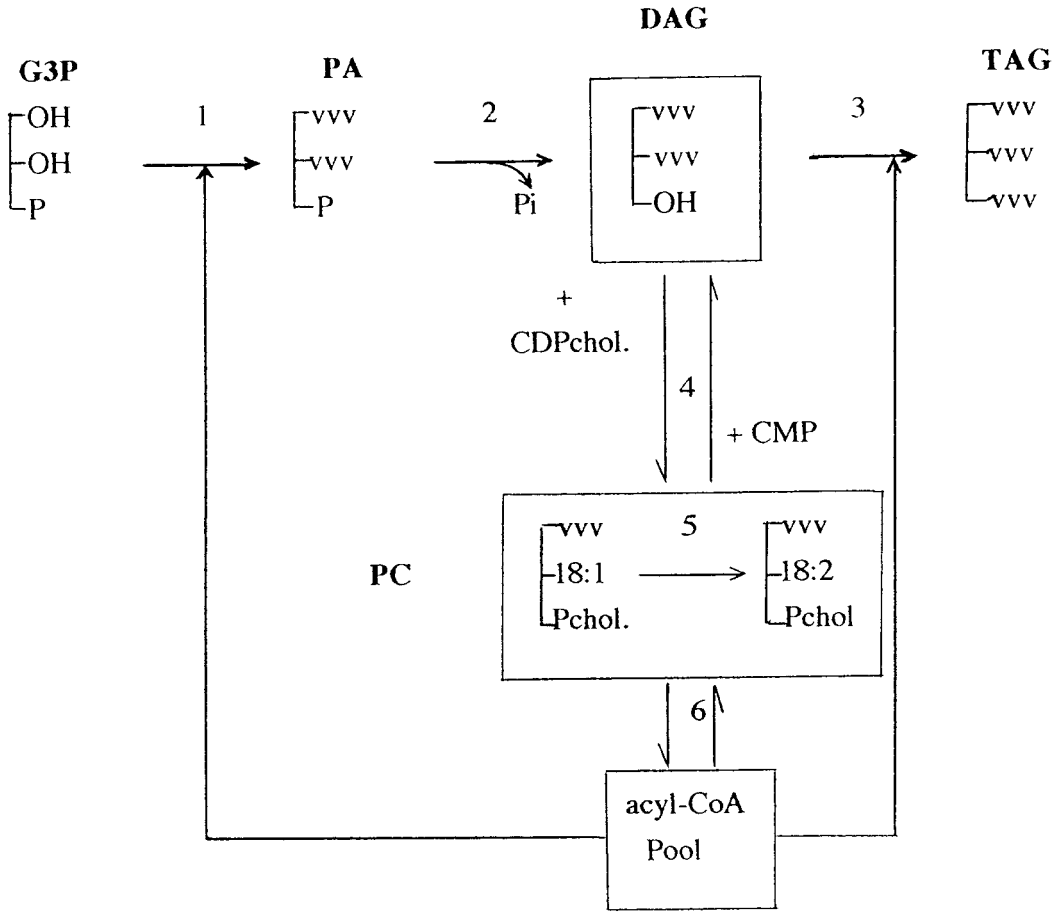


Fig. 3. Biosynthetic pathway of the TAG in the developing sunflower seeds. G3P, glycerol 3-phosphate, vvv: fatty acid. Enzymes for numbered reactions: (**1**) G3P and lyso PA-acyltransferases, EC 2.3.1.15 and 51; (**2**) phosphatidate-phosphatase, EC 3.1.3.4; (**3**) DAG-acyltransferase, EC 2.3.1.20; (**4**) CDP choline:DAG-phosphotransferase, EC 2.7.8.2; (**5**) oleoyl PC-desaturase; (**6**) lyso PC-acyltransferase, EC 2.3.1.23.

were ground in a waring blendor with 250 ml of 0.1 M Tris–HCl buffer, pH 7.5 and containing 10 mM KCl, 1 mM benzamidin. The homogenate was filtered through 4 layers of Miracloth and centrifuged at $10000 \times g$ for 15 min. The supernatant was filtered through Miracloth again then centrifuged at $100000 \times g$ for 90 min. The resulting microsomal pellet was rapidly resuspended in 0.1 M Pi buffer (pH 7.2) and stored at $-60°C$ until required.

4.4. Incubations

The incubation mixture contained labelled substrate: 51.8 nmol [1-^{14}C]oleic acid (specific radioactivity, 1.96 MBq mmol$^{-1)}$ or 45.7 nmol [1-^{14}C]linoleic acid (specific radioactivity 2.18 MBq mmol^{-1}). We added cofactors for fatty acid activation according to Macey (1983), 25 μM ATP, 20 μM CoA and 10 mM $MgCl_2$; 2 mM NADH if the marker was [^{14}C]oleate, 1% BSA to stimulate acylations (Ichihara, 1984), with the microsomal suspension (0.1 ml; 1.72 to 2.25 mg of protein as determined by Lowry, Rosenbrourgh, Farr, and Randall (1951)). The final volume was adjusted to 0.5 ml with 0.1 M Pi buffer pH 7.2. All assays were carried out at 30°C with constant shaking. Reactions were stopped by adding boiling MeOH.

4.5. Lipid extraction and analysis

Microsomal lipids were extracted following the Bligh and Dyer method (Bligh & Dyer, 1959). Lipid classes were separated by TLC using precoated silica-gel plates (Merck), with Mangold solvant (Mangold, 1964) to purify DAG and TAG and the Tremolières-Lepage solvant (Tremolières & Lepage, 1971) to obtain PC. Lipid areas were located by primuline (1 g l^{-1} in Me_2CO–H_2O, 80:20) under UV beams and were identified by comparison with commercial standards (Sigma) then scraped from the plates, either assayed for radioactivity or separated into molecular species by HPLC.

4.6. Molecular species analysis

Lipid classes separated by TLC were extracted from the silica gel with 5 ml of MeOH–HOAc (5:0.05) over

night. Then, 5 ml of $CHCl_3$ was added and 5 ml of H_2O. The lipids were recovered in $CHCl_3$, after evaporation in a stream of N_2, the volume was reduced to 1 ml and filtered on a Millipore (0.45 μm) filter then injected into the HPLC column as 20 μl fractions. In order to separate the molecular species of DAG by HPLC, we prepared the naphtylurethane derivatives of these molecules (Krüger, Rabe, Reichmann, & Rüstov, 1984): DAG extracted in $CHCl_3$ and evaporated to dryness were dissolved in 100 μl of dimethylformamide, 1 μmol of α-naphtylisocyanate and 0.2 μmol of diazabicyclo (2,2,2) octane, heating the solution in a stoppered vial at 85° for 2 h. After cooling to room temperature, the solution was diluted with 1 ml of methanol and filtered on a millipore filter.

Chromatographies were carried out on a Waters Associates (Milford, MA, USA) apparatus equipped with a variable wavelength detector. Two pumps (model 510) were combined with a model 680 automated gradient controller and a model U6K system injector. Lipid separation was achieved on a C_{18} reverse phase column (3.9 × 300 mm) packed with 10 μm Bondapack. The elution mixtures contained MeOH–H_2O–acetonitrile–choline chloride (90.5:7:2.5:0.28 g, v:v:v:w) for PC, according to Demandre, Tremolières, Justin, and Mazliak (1985) and (90.5:4:2.5:0.28 g, v:v:v:w) for DAG naphthylurethanes, as detailed in Justin, Demandre, Tremolières, and Mazliak (1985), at a flow rate of 1.5 ml min^{-1}. The two classes were detected at 205 and 290 nm, respectively. The molecular species of TAG were eluted in MeOH–acetonitrile–choline chloride (90:10:0.28 g, v:v:w) at a flow rate of 2 ml min^{-1} and detected at 210 nm.

4.7. Determination of lipid radioactivity

The labelled spots were scraped from the plates into vials for liquid scintillation counting (Kontron intertechnique, scintillator SL 3000) in a toluene solution (0.5% 2,5-diphenyl-1,3-oxazole and 0.03% 2,2′-phenylenbis 5-phenyloxazol). The radioactivity of each compound eluted from HPLC column was measured by scintillation in a continuous flow apparatus (Flow one IC Radiomatic instruments, in a Luma flow 2 solution). Data were processed using a Qume computer.

4.8. Fatty acid analysis

Fatty acid methyl esters from each molecular species collected at the exit of the HPLC column were prepared by transesterification with sodium methoxide (Carreau & Dubacq, 1978). Methyl esters were analysed by gas chromatography on a Carbowax capillary column (25 m × 0.4 mm) at 170°C, with helium as a carrier gas (flow rate, 40 ml min^{-1}). For quantification, an appropriate amount of an internal standard (heptadecanoic acid) was added to the pooled eluate representing each peak. TLC on $AgNO_3$-impregnated silica gel (Hulanicka, Erwing, & Bloch, 1964) was also used to separate methyl esters according to degree of unsaturation. Fatty acids were located under UV light, after spraying 2′,7′-dichlorofluorescein (0.2% in EtOH) and subsequent autoradiography (Kodak-Kodirex film).

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