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PHOSPHATIDYLCHOLINE AND ITS RELATIONSHIP TO TRIACYLGLYCEROL BIOSYNTHESIS IN OIL-TISSUES

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Key Word Index—*Carthamus tinctorius*; Compositae; safflower; *Cucurbita pepo*; Cucurbitaceae; marrow; *Persea americana*; Lauraceae; biosynthesis; oil tissues; triacylglycerols; phosphatidylcholine (glycerol-moiety); polyunsaturated fatty acids.

Abstract—During maturation of safflower cotyledons a marked change occurred in the pattern of [^{14}C] glycerol (G-OH) incorporation into the complex lipids. In the early stages of development, the G-OH-backbone of phosphatidylcholine (PC) with associated acyl-groups, is rapidly turned-over and channelled into the accumulating storage oils (triacylglycerol, TAG). In almost mature cotyledons, [^{14}C]G-OH accumulated in diacylglycerol (DAG). In contrast, the germinating seed cotyledons of marrow, despite active incorporation of [^{14}C]G-OH into TAG, principally synthesized PC which did not participate in oil synthesis. The role of the PC-DAG interconversion in generating polyunsaturated fatty acids, and thus affecting acyl-quality, is discussed.

INTRODUCTION

The biosynthesis of triacylglycerol (TAG) has been extensively studied in microsomal preparations from developing cotyledons of species with linoleate-rich oils [1]. The evidence suggests that such seeds possess two modes of entry of oleate into phosphatidylcholine (PC) for desaturation. One which involves an acyl exchange between acyl-CoA and position sn-2 of PC [2, 3] and the other, that involves diacylglycerol (DAG) [4–6]. In microsomal membranes from developing seeds of safflower, there is a rapid interconversion of DAG and PC pools during active TAG formation but without net synthesis of PC [6]. Thus, the entry (and exit) of acyl substrate (and product) that is facilitated by the acyl exchange and the interconversion of DAG and PC, would help to regulate the fatty acid quality of the final oil. Recently, we confirmed the importance of the turnover of acyl groups at position sn-2 of PC in providing C_{18}-polyunsaturated acyl substrates for TAG synthesis *in vivo* in tissue slices of maturing oil-seeds [7].

To add further credence to our proposals [1] it is also important to ascertain whether the turnover of the glycerol (G-OH)-backbone of PC that is observed *in vitro* [5, 6] and indicated from earlier *in vivo* studies [8] also occurs in the developing cotyledons of safflower and, if so, to what extent. However, to interprete, more fully, the role of PC in TAG formation, it was found necessary to carry out comparative studies and to establish the kinetic patterns of [^{14}C] G-OH utilisation in other species and in tissues of differing physiological states of development. Hence, the incorporation of [^{14}C]G-OH in slices of maturing safflower, at various stages of oil-deposition, has been compared with that in the germinating, fat-mobilising, seed cotyledons of marrow (*Cucurbita pepo*) and in avocado mesocarp (*Persia americana*), a tissue which *in vitro* in microsomal membranes appears to lack a significant DAG-PC interconversion [9]. It is these experiments that are reported here.

RESULTS

Utilisation of [U-^{14}C]G-OH *by maturing cotyledons of safflower*

Maturing cotyledons of safflower were harvested from three developmental stages. Namely, 14–16 days after flowering (DAF), (corresponding to 15–20% of the total oil deposited), 20–22 DAF (75–80% of the final oil content) and 26–28 DAF (almost 100% oil deposition). The uptake, and subsequent incorporation of [^{14}C]G-OH into the lipid fraction, was comparable in all three developmental stages and entered the lipids at a linear rate over the first 80 min of incubation and, thereafter, more slowly (data not shown). It was, therefore, possible to compare the amount (nmol of [^{14}C]G-OH) of radioactivity which entered the complex lipids. The incorporation of radioactivity from [U^{14}C]G-OH into the cotyledon lipids at 14–16 DAF was predominantly in PC, DAG and TAG (Fig. 1A). These lipids contained between 87 and 94% of the total radioactivity in the lipid fraction with most of the remainder present in phosphatidylethanolamine (PE). PC was rapidly labelled initially and continued to accumulate radioactivity up to 40 min and thereafter remained almost constant. DAGs were labelled in a similar manner to PC although they contained somewhat lower levels of radioactivity at all time periods. The accumulation of [^{14}C]G-OH in TAG, however, was slow over the first 20 min and then increased at an accelerating rate becoming the most radioactive lipid after 40 min. Between 40 and 80 min the radioactivity in

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TAG doubled and at the end of the incubation it contained 63% of the total lipid radioactivity whereas PC and DAG contained 18 and 10%, respectively.

Studies on the utilization of [U-^{14}C]G-OH by safflower cotyledons harvested 20–22 DAF (Fig. 1B), showed that both DAG and PC were labelled at a linear rate while TAG accumulated radioactivity at an accelerating rate. A comparison of the mass of [^{14}C]G-OH incorporated into TAG by the 14–16 DAF and the 20–22 DAF cotyledons after 160 min of incubation shows that almost five times more G-OH was incorporated into this lipid by the younger cotyledons.

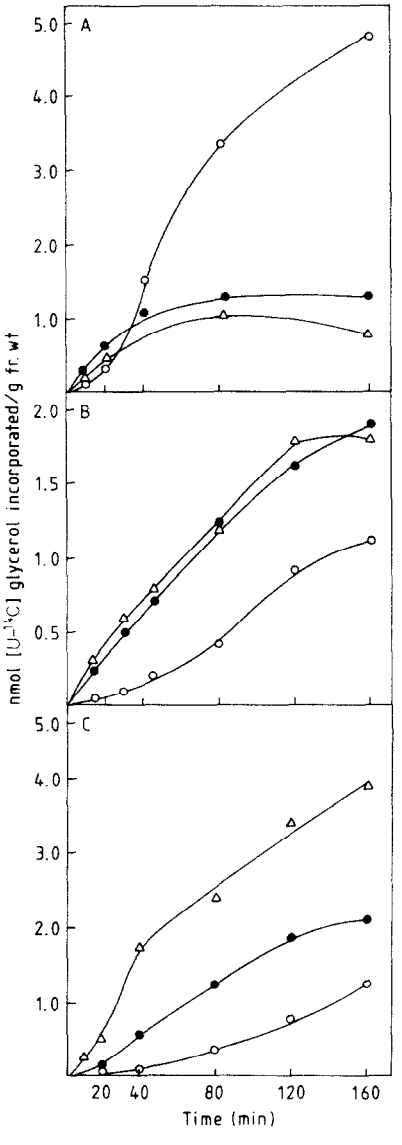

Fig. 1. Incorporation of [U^{14}C]G-OH into the major lipids of developing safflower cotyledons harvested (A) 14–16 DAF, (B) 20–22 DAF and (C) 26–28 DAF. Tissue slices of the cotyledons (equivalent to 0.3 g fr.wt) were maintained in buffered solutions containing 74 kBq of [U-^{14}C]G-OH. At regular intervals, the lipids were extracted and radioassayed as described in the Experimental. ●, PC;Δ, DAG; ○, TAG.

The results obtained from supplying [U-^{14}C]G-OH to the almost mature cotyledons (harvested 26–28 DAF, Fig. 1C) again shows a different pattern of incorporation to that found in the younger cotyledons. One of the major differences was the dissimilarity in the labelling of PC and DAG. The DAGs contained the highest proportion of the [^{14}C]G-OH throughout the experiment while the amount of radioactivity in PC was some 50% of this value at almost all of the incubation times. The TAGs, on the other hand, became labelled at an accelerating rate after 40 min at which time the rate of labelling of DAG started to decline. The amount of radioactivity which had accumulated in TAG at the end of the incubation, however, was similar to that observed in the cotyledons harvested 20–22 DAF.

The results indicate that the most rapid rate of G-OH incorporation into TAG occurred in the youngest cotyledons harvested 14–16 DAF. In order to assess, therefore, whether the PC-backbone could contribute to TAG accumulation, 'pulse-chase' experiments were performed with cotyledons at this stage of seed development. Cotyledon samples were pre-incubated in [^{14}C]G-OH for 30 min and, after thorough washing, placed in fresh buffer containing 5 mM non-radioactive G-OH. A typical 'chase' experiment is illustrated in Fig. 2. The results are expressed on a relative basis since the total radioactivity in the lipid fraction increased (and correspondingly decreased in the aqueous fraction) in the tissue over the first 40 min following the transfer to the non-radioactive substrate (data not shown). The radioactivity in the PC backbone rapidly declined and there was a concomitant increase in TAG. After 40 min, however, the total ra-

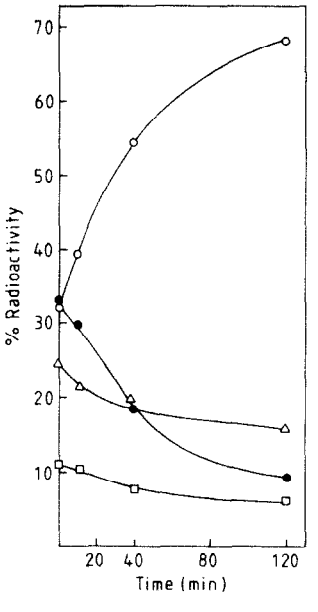

Fig. 2. Redistribution of [U-^{14}C] G-OH between the major lipid classes in developing safflower cotyledons (14–16 DAF) following the transfer to an unlabelled medium. Tissue slices of safflower were preincubated with [U-^{14}C] G-OH for 30 min, washed with distilled water, and then incubated further in buffered unlabelled G-OH (5 mM) for the times indicated above. Lipids were extracted and radioassayed as described in the Experimental. ●, PC; Δ, DAG; ○, TAG; □, other lipids.

Table 1. Change in specific radioactivity of major acyl lipids in safflower cotyledons labelled from [^{14}C]G-OH

Time in unlabelled G-OH (min)	Specific radioactivity (kBq/nmol) PC	DAG	TAG
40	2.35	2.82	0.25
120	1.34	2.42	0.30

dioactivity in the lipid fraction remained almost constant and calculations, based on the change in the specific radioactivity of the lipids over this time period (Table 1), indicated that some 40% of the [^{14}C]G-OH in the PC pool had been displaced in the direction of TAG production. The small but consistent increase in the specific radioactivity of the triacylglycerols can be fully accounted for on the basis of the larger mass of this lipid in the cotyledons (mass ratio of TAG/PC was 25). Calculations based on the mass of [^{14}C]G-OH in each lipid showed that over 70% of the increase in TAG could be attributable to the decrease in PC and the remainder from DAG. Similar results were obtained with safflower cotyledons harvested at maturity, although the phosphatidylcholine-backbone was turned over more slowly and was transferred to TAG only after prolonged incubation times (data not shown).

Utilization of [U-^{14}C]G-OH *by etiolated cotyledons of germinating marrow*

Tissue slices of marrow cotyledons were supplied with [U-^{14}C]G-OH in experiments similar to those reported above for safflower. The results obtained from one such experiment are presented in Fig. 3. During the 4 hr incubation period, [^{14}C]G-OH entered the lipid fraction at a

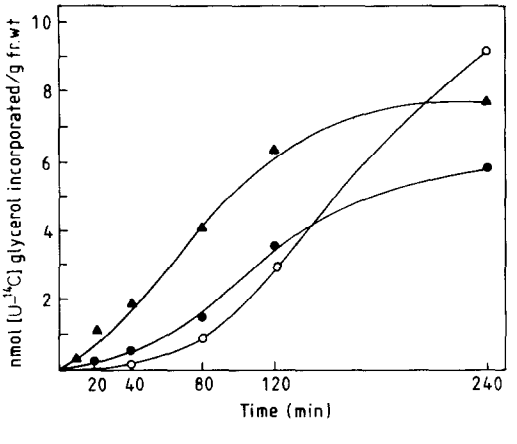

Fig. 3. Incorporation of [U-^{14}C] G-OH into lipids by tissue slices of seven-day dark grown *Cucurbita pepo*. Cotyledons were maintained in buffered solutions containing [U-^{14}C] G-OH and the lipids were extracted at regular intervals. ●, PC; ▲, DAG; ○, TAG.

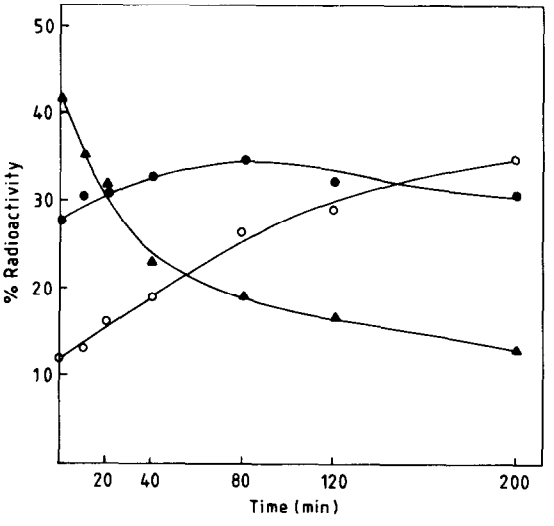

Fig. 4. Redistribution of [U-^{14}C] G-OH between the major lipid classes in seven day dark grown *Cucurbita pepo*. Cotyledon slices were incubated in U-^{14}C G-OH for 80 min and then transferred to an unlabelled media containing 5 mM G-OH. Lipids were extracted as described in the Experimental. ●, PC; ▲, DAG; ○, TAG.

rate that was linear with time (data not shown). The DAGs were also labelled at a linear rate from zero time up to 2 hr and more slowly thereafter. PC and TAG, however, were both labelled at accelerating rates with radioactivity rapidly accumulating in the latter lipid. After 4 hr of incubation, TAG was the most radioactive lipid and contained some 30% of the total label while DAG and PC contained 25 and 18%, respectively. Most of the remaining radioactivity was present in PE and the chloroplast lipids. To determine whether the G-OH backbone of PC could be utilized for TAG synthesis, the cotyledons were incubated with [U-^{14}C]G-OH for 80 min as previously described (see legend to Fig. 3) and then 'chased' with non-radioactive G-OH (5 mM) for a further 200 min (Fig. 4). During the 'chase' period the total radioactivity in the lipids remained almost constant (20 ± 1 nmol U-^{14}C]G-OH/g fr. wt). The results, therefore, presented in Fig. 4, clearly demonstrate the movement of [^{14}C]G-OH in DAG to TAG. Corresponding changes in PC, however, were negligible.

Utilization of [U-^{14}C]*G-OH by mature avocado mesocarp*

The amount of [U-^{14}C]G-OH taken up by tissue slices of avocado was always found to be somewhat variable in time-course studies. Generally, however, the amount of radioactivity incorporated into the lipid fraction was only some 30% of that observed for the safflower cotyledons. Typical results, therefore, from one such experiment are presented on a relative basis (see Fig. 5). Troughout the time-course [^{14}C]G-OH was present in DAG and TAG and after 80 min of incubation these lipids contained 30 and 60% of the total radioactivity, respectively. PC, on the other hand, contained less than 3% of the radioactivity in the lipid fraction throughout the entire experiment.

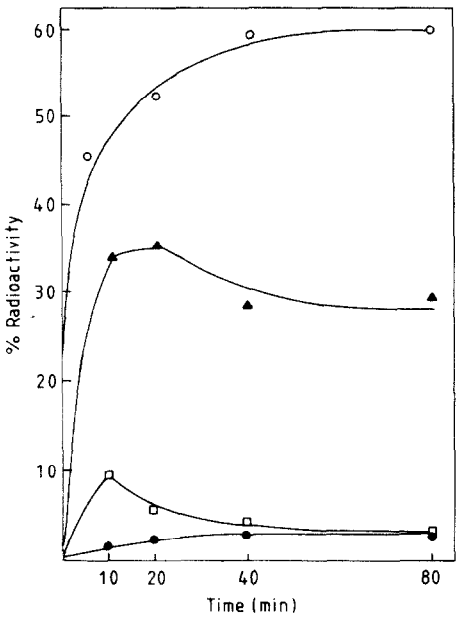

Fig. 5. Incorporation of [U-^{14}C] G-OH into avocado mesocarp lipids. Tissue slices of the inner mesocarp (equivalent to 0.3 g fr. wt) were continuously incubated in buffered solutions containing 74 kBq [U-^{14}C]G-OH for the times indicated. Lipids were extracted as described in the Experimental. ●, PC; □, phosphatidic acid; ▲, DAG; ○, TAG.

DISCUSSION

During maturation of safflower seeds a marked change occurred in the pattern of incorporation of [^{14}C]G-OH into the complex lipids. In the youngest cotyledons in which oil deposition was rapid (Fig. 1A), the G-OH moiety of PC was rapidly turned-over and utilized in TAG synthesis. This observation is consistent with the current proposal that the PC and DAG pools are in equilibrium, a reaction that may be catalysed by a cholinephosphotransferase [5, 6, 8]. With seed maturation the incorporation of [^{14}C]G-OH into TAG decreased and more radioactivity became associated with PC and DAG (Fig. 1C). Such a marked change in the labelling kinetics of these lipids during seed development may be attributable to a decrease in the activity of DAG acyltransferase and in the rate of the back reaction of the cholinephosphotransferase. Evidence for changes in the activity of these enzymes in maturing oil tissues has also been observed in *in vitro* studies with *Cocoa* [11] and safflower [5]. It was, however, perhaps surprising to observe extensive incorporation of [^{14}C] G-OH into TAG in the germinating and fat mobilising cotyledons of marrow. It is interesting that the overall labelling pattern of the lipids in the marrow was similar to that found in the most mature stage of seed development in safflower (cf. Figs 1C and 3). Despite the extensive labelling of TAG in the germinating seeds, there was, however, little or no contribution to this lipid from the G-OH backbone of PC. The incorporation of [^{14}C]G-OH into PC in marrow would be consistent with its net *denovo* synthesis for membrane assembly during seed germination. In avocado, despite a substantial pool of endogenous PC potentially available for participation in TAG production, the phospholipid was poorly labelled throughout the time-course even though DAG and TAG were extensively synthesized. This data substantiates earlier studies using isolated microsomal membranes from avocado [9, 12], where the possibility of cholinephosphotransferase loss or inactivation could not be ruled out as an explanation for the lack of G-OH incorporation into PC.

The present data illustrate, therefore, three distinctly different utilization patterns for [^{14}C]G-OH in complex-lipid assembly in physiologically differing tissues. (i) A high flux of G-OH through PC into TAG (developing safflower cotyledons). (ii) Net incorporation of G-OH into PC with low turnover (marrow). (iii) Low incorporation of G-OH into PC at all times (avocado). *In vivo*, therefore, in developing cotyledons of safflower, and in other species, e.g. linseed [8] and sunflower [13] there appears to operate a system which equilibrates DAG and PC during active TAG deposition. Such a reaction may help bring about further enrichment of the glycerol-backbone with C_{18}-polyunsaturated fatty acids, which are synthesized on the PC [5, 14] and so play some role in regulating the acyl quality of the oil. In view of the amount of linoleate in marrow and avocado triacylglycerols (60 and 18%, respectively, [13]) it is also possible that a similar mechanism may be operative in these tissues during their natural development at the time of most active oil deposition. Whether the DAG-PC equilibration is catalysed by a cholinephosphotransferase in the oil-seeds requires further study, particularly on the properties of the enzyme(s) which appear to allow reversibility during TAG formation in developing C_{18}-polyunsaturated oil-seeds and yet in others circumstances (seed germination) functions in the net synthesis of PC.

EXPERIMENTAL

Safflower plants (*Carthamus tinctorius* L, var. Very High-Linoleate) were grown from seed in a 16-hr photoperiod at a temp of 28–32° and 8-hr night with a minimum temp of 20°. Vegetable marrow (*Cucurbita pepo* L., cv. Long Green Trailing) plants were grown from seed in the dark at 25°. The etiolated cotyledons were harvested after 7-days dark growth for experimental purposes. Avocados (*Persea americana*) were purchased locally. Experimental tissue was always from freshly harvested material and was used within 0.5–1 hr after excision. Expts were performed on at least two occasions with different batches of seed or mesocarp material and all gave similar results.

Incubations were carried out in test tubes at 30° and with constant shaking. Each incubation sample contained *ca* 0.3 g fr. wt of thinly-sliced tissue and [U-^{14}C]G-OH (equivalent to 74 kBq) in a total vol of 1 ml K-Pi buffer, pH 7.2. After incubation, cotyledons were throughly rinsed with H_2O prior to lipid extraction (or transfer to a medium containing non-radioactive substrate) in order to remove exogenougly bound isotope. The lipids were extracted from the tissues by homogenisation in $CHCl_3$–MeOH–0.15 MHOAc (4:8:3, 37.5 ml) followed by the further addition of $CHCl_3$ (10 ml) and H_2O (10 ml), modified from ref. [10]. The $CHCl_3$ phase, which contains the complex-lipids and unesterified fatty acids, was reduced to dryness under N_2. The residue was dissolved in a small vol. of $CHCl_3$ and the polar and neutral lipids purified by TLC on precoated silica gel plates (silica gel 60, Merck) with $CHCl_3$–MeOH–HOAc–H_2O (170:30:20:7) and hexane–Et_2O–HOAc (70:30:1), respectively. Lipids were located by lightly staining with I_2 vapour and then

removed from the plates for liquid scintillation radioassay in PCS (Amersham/Searle, Arlinton Heights, U. S. A.)/xylene (2:1). Counts were corrected for quenching. Between 90 and 95% of the radioactivity from [U-^{14}C]G-OH was accounted for in the G-OH moieties of the acyl lipids.

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