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# Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor-bean (*Ricinus communis*) endosperm

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Microsomal membrane preparations from the developing endosperm of castor bean (*Ricinus communis*) catalysed the transfer of oleate from [$^{14}$C]oleoyl-CoA to phosphatidylcholine (PtdCho). In the presence of NADH, radioactive ricinoleate (12-hydroxyoctadec-9-enoate) was synthesized from [$^{14}$C]oleate, and this was largely recovered in PtdCho and as free fatty acid. The addition of unlabelled ricinoleoyl-CoA to these incubation mixtures did not increase the low [$^{14}$C]ricinoleate concentration found in the acyl-CoA fraction nor decrease the [$^{14}$C]ricinoleate concentration in PtdCho and free fatty acid, and thus no evidence was obtained for a hydroxylation with oleoyl-CoA as a substrate. The addition of NADH, necessary for the formation of ricinoleate, caused a decrease of the total radioactivity in PtdCho with a corresponding increase in the amount of label in free ricinoleic acid. This increase was due to the action of a phospholipase A, which released ricinoleic acid but not oleic acid from PtdCho. Such a phospholipase activity, attacking ricinoleoyl-PtdCho but not oleoyl-PtdCho, was also demonstrated in microsomal preparations from developing cotyledons of safflower and oil-seed rape. An analysis of the acyl groups at different positions in microsomal PtdCho of castor bean showed that ricinoleate was almost entirely associated with position *sn*-2. Likewise the [$^{14}$C]ricinoleate in [$^{14}$C]PtdCho formed after incubations with microsomal preparations with NADH and [$^{14}$C]oleoyl-CoA resided in position *sn*-2 with none in position *sn*-1. In contrast, the [$^{14}$C]linoleate formed by desaturation of [$^{14}$C]oleoyl-PtdCho was present at both positions. In the presence of ATP, CoA and $Mg^{2+}$, the ricinoleate acid released from PtdCho was activated to ricinoleoyl-CoA. The ricinoleoyl-CoA was an efficient acyl donor in the acylation of glycerol 3-phosphate (Gro3*P*) to yield phosphatidic acid and triacylglycerols. In microsomal preparations incubated with an equimolar mixture of [$^{14}$C]oleoyl-CoA and [$^{14}$C]ricinoleoyl-CoA in the presence of Gro3*P*, only a minor amount of [$^{14}$C]ricinoleate entered PtdCho, and this was believed to be via the exchange of phosphocholine groups between a diacylglycerol pool and the PtdCho. On the basis of our results, a scheme of ricinoleate formation and its incorporation into triacylglycerols in castor-bean endosperm is proposed.

## INTRODUCTION

Vegetable oils rich in ricinoleic acid (12-hydroxyoctadeca-9-enoic acid) are of particular interest to the oleochemical industry (Pryde & Rothfus, 1989). Few plants species, however, accumulate triacylglycerols (triacylglycerides) with hydroxylated fatty acids. The only commercial source is from castor-bean (*Ricinus communis*) endosperm, in which over 80% of the fatty acid in the triacylglycerol is ricinoleic acid. Earlier work (Galliard & Stumpf, 1966), with cell-free and microsomal membrane preparations from developing castor beans, showed that oleate ($C_{18:1,\Delta9}$) was the acyl substrate for the hydroxylation and that the reaction required NAD(P)H. Kinetic data (Moreau & Stumpf, 1981), on ricinoleate formation from exogenously supplied [$^{14}$C]oleoyl-CoA, were interpreted as indicating that the oleate was esterified to microsomal phosphatidylcholine (PtdCho) before hydroxylation. In these experiments, however, little [$^{14}$C]ricinoleate was found in PtdCho, the major portion being present in an undefined 'non-polar' fraction. Questions, therefore, regarding the immediate substrate for the $\Delta^{12}$-fatty acid hydroxylase and the assembly of triacylglycerol with hydroxy fatty acids remain largely unresolved (Stymne & Stobart, 1987). Here we present evidence on the nature of the lipid substrate for the hydroxylase enzyme and investigate ricinoleate utilization in triacylglycerol formation in microsomal preparations from the developing endosperm of castor bean.

## MATERIALS AND METHODS

### Chemicals

[1-$^{14}$C]Oleic acid and *sn*-[U-$^{14}$C]glycerol 3-phosphate ([U-$^{14}$C]Gro3*P*) were obtained from Amersham International, Amersham, Bucks., U.K. Catalase, BSA (fraction V, fatty acid-free), triacylglycerol lipase (*Rhizopus arrhizus*), CoA, Gro3*P*, lysophosphatidylcholine (*sn*-1-acylglycero-3-phosphocholine) (lysoPtdCho) (prepared from egg yolk), oleic acid and ricinoleic acid were purchased from the Sigma Chemical Co.

[1-$^{14}$C]Ricinoleic acid was synthesized enzymically by hydroxylation of [1-$^{14}$C]oleate by microsomal preparations from developing castor-bean endosperm as follows. Microsomal preparation (10 mg of microsomal protein) was incubated for 4 h at 25 °C with 1 μmol of [1-$^{14}$C]oleic acid (sp. radioactivity 52 μCi/μmol) bound to 80 mg of BSA, 8000 units of catalase, 15 μmol of NADH, 16 μmol of ATP, 18 μmol of $MgCl_2$ and 8 μmol of CoA in 0.1 M-potassium phosphate buffer, pH 7.2, in a total volume of 8 ml. The lipids were saponified by heating the mixture in 0.5 M-KOH at 100 °C for 60 min. After acidification with HCl and extraction with chloroform, the fatty acids were esterified with

Abbreviations used: Gro, glycerol; Gro3*P*, *sn*-glycerol 3-phosphate; lysoPtdCho, lysophosphatidylcholine (*sn*-1-acylglycero-3-phosphocholine); PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid.

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methanolic HCl (Kates, 1964). The methyl ricinoleate was separated from the non-hydroxylated methyl esters by t.l.c. on silica gel 60 with hexane/diethyl ether/acetic acid (70:30:1, by vol.). The area corresponding to methyl ricinoleate was removed from the plates and the lipid was eluted from the gel with chloroform/methanol (2:1, v/v). The solvent was evaporated, and the methyl esters were saponified with 1 ml of 2 M-KOH at 100 °C for 60 min. After acidification with HCl, the free [$^{14}$C]-ricinoleic acid was extracted in chloroform. The yield of [$^{14}$C]-ricinoleate was about 20% of added radioactivity and the final specific radioactivity was 12500 d.p.m./nmol. Radiolabelled and non-labelled oleoyl-CoA and ricinoleoyl-CoA were synthesized from their mixed anhydrides (Sánchez *et al.*, 1973; Stobart & Stymne, 1990).

### Plant material and microsomal preparation

Microsomal preparations from developing cotyledons of oilseed rape (*Brassica napus* var. Topas) and safflower (*Carthamus tinctorius* var. Gila) were prepared by the procedure of Stobart & Stymne (1990). Castor bean (*Ricinus communis* var. Rogusus) plants were grown from seed in a 16 h photoperiod at 25 °C and 8 h night at 20 °C. Developing seeds were harvested between 30 and 40 days after flowering and cut into two parts. Only those seeds where the endosperm had expanded to between one-third and two-thirds of the seed volume were used for microsomal preparation. The endosperm tissues, together with the small embryo, were removed from the seed and stored on ice. All further manipulations were carried out at 0 °C. The tissue was ground in a mortar with 5 parts (v/w) of 0.1 M-potassium phosphate buffer, pH 7.2, containing 0.1% BSA, 1000 units of catalase/ml 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, containing 0.1% of BSA and 1000 units of catalase/ml. The microsomal pellets were used either fresh or stored up to 2 weeks at −80 °C. Longer storage periods greatly decreased the oleate hydroxylation activity of the preparation.

### Protein determination

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

### Assays

Microsomal incubations with radioactive substrates were carried out with constant shaking at 25 °C and with acyl-CoAs, acyl acceptors, cofactors and microsomal membranes (as given in the Table and Figure legends) and 1000 units of catalase in a final volume of 1 ml with 0.1 M-sodium phosphate buffer, pH 7.2. Reactions were terminated by the addition of 1 ml of 0.15 M-acetic acid, and the complex lipids and non-esterified fatty acids were extracted by the 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).

### Analytical procedures

When the analysis of the acyl groups in the acyl-CoA fraction was required, the upper methanol/water phase (into which the acyl-CoA partitions) from the Bligh & Dyer (1959) extraction were extracted further with an additional 2.5 ml of chloroform. The lower chloroform phases were bulked and used for the analysis of complex lipids and non-esterified fatty acids as described below. Acyl-CoA in the aqueous-methanol phase was hydrolysed by the addition of 2 ml of 2 M-KOH and refluxed for 40 min. After acidification with HCl, the resulting non-esterified fatty acids were extracted in chloroform. After evaporation of the chloroform under $N_2$, the fatty acids were methylated with methanolic HCl (Kates, 1964) and, after the addition of methyl-heptadecanoic acid as internal standard, analysed by g.l.c. and radio-g.l.c. Although all the acyl-CoA was recovered by this method, a minor part of the microsomal lipids also partitioned into the upper phase (1–2% of total acyl groups in the microsomal membranes).

Complex lipids and non-esterified fatty acids in the chloroform phase were evaporated to dryness under $N_2$. The residue was dissolved in a small volume of chloroform, and the polar 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.). Neutral lipids were purified on high-performance t.l.c. plates (Merck; silica gel 60) in hexane/diethyl ether/acetic acid (70:140:3, by vol.).

The presence of ricinoleoyl groups in complex lipids affected their mobility in the t.l.c. system. The presence of ricinoleate in the polar lipids had much less influence on the migration rate than the ricinoleate in neutral lipids. PtdCho with one ricinoleoyl group co-chromatographed with PtdCho without hydroxylated acyl groups, whereas phosphatidic acid (PtdOH) with one ricinoleoyl moiety was only partially resolved from PtdOH with two ricinoleoyl groups and with no ricinoleate. In the neutral lipids (i.e. the non-esterified fatty acid, monoacylglycerols, diacylglycerols and triacylglycerols) the presence of ricinoleate dramatically altered their chromatographic behaviour. The use

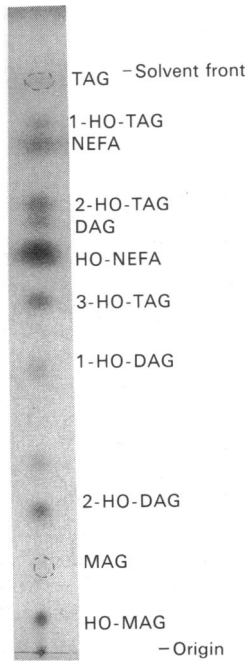



**Fig. 1. T.l.c. chromatogram of ricinoleate neutral lipids**

The lipids were chromatographed on high-performance silica-gel t.l.c. plates in hexane/diethyl ether/acetic acid (70:140:3, by vol.) and stained with $I_2$ vapour. The lipid mixture was obtained by triacylglycerol lipase treatment of diricinoleoyl-containing triacylglycerol as described in the Materials and methods section and supplemented with dioleoylglycerol and oil from mature castor beans. Abbreviations: MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; NEFA, non-esterified fatty acid. The numbering designates mol of ricinoleate/mol of lipid (i.e. 2-HO-TAG, triacylglycerols with two ricinoleoyl moieties).

of high-performance silica-gel t.l.c. plates in the solvent described above enabled the resolution of all these lipids according to the number of hydroxylated acyl groups present in each lipid class (Fig. 1). The different hydroxylated lipids were identified by first analysing the acyl composition of the three major lipid areas on a t.l.c. chromatogram of castor-bean oil. The proportion of ricinoleoyl groups in the three areas were (in order of decreasing mass in each area) 100, 66 and 33% of total acyl groups, and thus they consisted of triacylglycerols with three, two and one ricinoleoyl group respectively. Triacylglycerols with two ricinoleoyl groups were eluted from the gel and partially hydrolysed with *Rhizopus arrhizus* lipase by the procedure of Bafor *et al.* (1990). After chromatography of the hydrolytic products an additional five lipid spots were observed. Two were identified as free ricinoleic acid and free non-hydroxylated fatty acids, and the others, with a migration value less than the triacylglycerols substrate, were on the basis of ricinoleate content tentatively identified as ricinoleoyl-containing monoacylglycerols, ricinoleoyl/ricinoleoyl-containing diacylglycerols and ricinoleoyl/non-hydroxylated acyl-containing diacylglycerol.

Lipid areas were removed from the plates and either assayed for radioactivity or esterified *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.

Positional analysis of the PtdCho was performed after treatment of the lipid with phospholipase $A_2$ as described by Griffiths *et al.* (1985).

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

## RESULTS

### Hydroxylation of [$^{14}$C]oleate

Microsomal preparations from developing castor-bean endosperm were incubated with [$^{14}$C]oleoyl-CoA and NADH and the distribution of radioactivity in complex lipids and non-esterified fatty acids was determined (Table 1). In the absence of NADH the oleate was transferred largely to PtdCho with little radioactivity in the non-esterified fatty acids. The addition of NADH, however, resulted in the appearance of the radioactive free ricinoleic acid and with a corresponding decrease in the label in PtdCho. Time-course studies on the incorporation of radioactivity from [$^{14}$C]oleoyl-CoA into lipid species in the presence of NADH showed (Fig. 2) that [$^{14}$C]oleate from [$^{14}$C]oleoyl-CoA was mainly transferred to PtdCho. Total amount of [$^{14}$C]-ricinoleate increased throughout the time course. Similar quantities of [$^{14}$C]ricinoleate accumulated in PtdCho and free fatty acid up to 30 min of incubation, after which the amount remained constant in PtdCho but continued to increase in the free acid. It should be noted that after 60 min incubation 80% of the [$^{14}$C]ricinoleate in the chloroform-soluble lipids resided in free fatty acid. In contrast, [$^{14}$C]linoleate, formed by $\Delta^{12}$-fatty acid desaturase activity, was almost totally associated with PtdCho (Fig. 2*a*). The accumulation of [$^{14}$C]oleate as free fatty acid was only 25% of that found for free [$^{14}$C]ricinoleic acid (results not shown).

In order to establish if oleoyl-CoA could serve as a direct substrate for the hydroxylase, relatively high concentrations of unlabelled ricinoleoyl-CoA were added to microsomal membrane preparations incubated with [$^{14}$C]oleoyl-CoA and NADH and the specific radioactivity of the ricinoleate in the lipid fractions was determined. The rationale for this experiment was that, if ricinoleoyl-CoA were the product of hydroxylation, the unlabelled ricinoleoyl-CoA should dilute substantially the specific radioactivity of the [$^{14}$C]ricinoleoyl-CoA formed from [$^{14}$C]-oleoyl-CoA. This would drastically decrease the [$^{14}$C]ricinoleate in the chloroform-soluble lipids (PtdCho and non-esterified fatty acids) and correspondingly greater amounts of [$^{14}$C]ricinoleate should reside in the acyl-CoA fraction. The results (Table 2), however, showed that the addition of unlabelled ricinoleoyl-CoA somewhat increased the [$^{14}$C]ricinoleate in PtdCho and free fatty acid and decreased the small amount of [$^{14}$C]ricinoleate in the acyl-CoA fraction. It should be noted that some two-thirds of the

**Table 1. Effect of NADH on the distribution of [$^{14}$C]acyl groups in lipids in microsomal preparations of developing castor-bean endosperm incubated with [$^{14}$C]oleoyl-CoA**

Microsomal preparations (equivalent to 0.9 mg of microsomal protein) were incubated with [$^{14}$C]oleoyl-CoA (50 nmol; sp. radioactivity 4500 d.p.m./nmol) and 10 mg of BSA in the presence and in the absence of NADH for 45 min. Results are the means ± S.E.M. for two incubations.

| | | [$^{14}$C]Acyl distribution (nmol) | | |
|---|---|---|---|---|
| | | | Free fatty acid | |
| NADH | Chloroform-soluble lipids (nmol) | PtdCho | Total | Ricinoleic acid |
| − | 28.38 ± 0.05 | 17.54 ± 0.02 | 2.83 ± 0.50 | 0 |
| + | 27.76 ± 1.88 | 9.14 ± 0.57 | 10.87 ± 1.12 | 9.49 ± 1.38 |

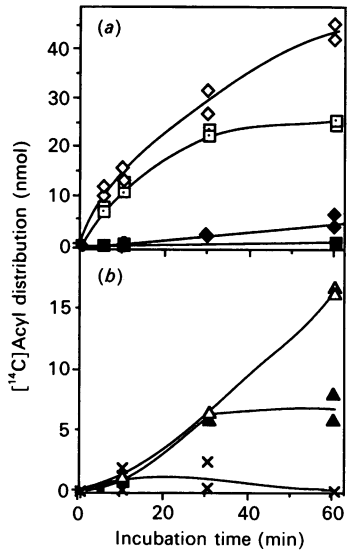



**Fig. 2. Effect of NADH on the utilization of [$^{14}$C]oleoyl-CoA by microsomal preparations of developing castor-bean endosperm**

Microsomal preparations (equivalent to 0.5 mg of microsomal protein) were incubated with [1-$^{14}$C]oleoyl-CoA (100 nmol; sp. radioactivity 4500 d.p.m./nmol) in the presence of 7.5 mg of BSA, 3 μmol of NADH and 1000 units of catalase in 0.1 M-phosphate buffer, pH 7.2, in a total volume of 1 ml at 25 °C. The radioactive incorporation into lipids and their fatty acids was determined after defined times. Symbols: ◇, oleate in chloroform-soluble lipids; ⊡, oleoyl-PtdCho; ◆, linoleoyl-PtdCho; ■, linoleate in chloroform-soluble lipids other than PtdCho; ▲, ricinoleoyl-PtdCho: △, free ricinoleic acid; ×, ricinoleate in chloroform-soluble lipids other than PtdCho and non-esterified fatty acids;

added ricinoleoyl-CoA still remained at the end of the incubation period.

Castor-bean microsomal membranes efficiently catalysed the acylation of exogenously supplied lysoPtdCho with ricinoleate from [$^{14}$C]ricinoleoyl-CoA (see below and Fig. 4). The acylation of PtdCho *in vivo* in oil-seeds, however, may occur largely via an acyl-exchange between an acyl-CoA pool and position *sn*-2 of *sn*-PtdCho (Stymne & Stobart, 1984, 1987). This reaction was therefore studied in castor-bean microsomal preparations incubated with oleoyl-CoA and ricinoleoyl-CoA as single and mixed acyl-CoA substrates and in the absence of added lyso-PtdCho. The results (Fig. 3) showed that the ricinoleate was incorporated into PtdCho but at a much lower rate (20%) than observed for the oleate. Little incorporation of [$^{14}$C]ricinoleate into PtdCho was observed in incubations with mixed acyl-CoA substrates, in which [$^{14}$C]ricinoleoyl-CoA and unlabelled oleoyl-CoA were provided in equimolar amounts (Fig. 3).

The *sn*-distribution of fatty acids in *sn*-PtdCho before and after a 60 min incubation of microsomal membranes with [$^{14}$C]-oleoyl-CoA and NADH was determined (Table 3). The *sn*-PtdCho in pre-incubation membranes contained some 9% ricinoleate in position *sn*-2, whereas this acid was only present in trace amounts at position *sn*-1. After incubation the mass of ricinoleate had increased to 20% and 3% at positions *sn*-2 and *sn*-1 respectively. Over 84% of the radioactivity incorporated into sn-PtdCho during incubation was confined to position *sn*-2, of which 46% was in ricinoleate. No radioactive ricinoleate was detected in position *sn*-1, whereas substantial [$^{14}$C]linoleate was present at both the *sn*-1 and *sn*-2 positions.

### Metabolism of PtdCho

The results described strongly indicate that the $\Delta^{12}$-fatty acid hydroxylase uses as substrate the oleate at position *sn*-2 of microsomal sn-PtdCho. The major proportion of synthesized ricinoleate at prolonged incubation times, however, was found to reside in free ricinoleic acid. These observations imply the

**Table 2. Effect of ricinoleoyl-CoA on the distribution of [$^{14}$C]ricinoleate in lipids in microsomal preparations from developing castor-bean endosperm incubated with [$^{14}$C]oleoyl-CoA and NADH**

Microsomal preparations (equivalent to 0.6 mg of microsomal protein) were incubated with [$^{14}$C]oleoyl-CoA (100 nmol; sp. radioactivity 4500 d.p.m./nmol), 3 $\mu$mol of NADH and 20 mg of BSA in the presence and in the absence of ricinoleoyl-CoA (300 nmol) for 30 min. Results are the means ± S.E.M. for two incubations.

| | Acyl groups recovered (nmol) | |
|---|---|---|
| Ricinoleoyl-CoA added... | − | + |
| Ricinoleate in acyl-CoA | 4.7 ± 0.3 | 207.8 ± 11.4 |
| [$^{14}$C]Ricinoleate in acyl-CoA | 3.83 ± 0.83 | 0.96 ± 0.19 |
| [$^{14}$C]Acyl groups in chloroform-soluble lipids | 51.0 ± 2.4 | 57.18 ± 3.8 |
| [$^{14}$C]Ricinoleate in PtdCho | 8.79 ± 0.82 | 13.4 ± 0.45 |
| Free [$^{14}$C]Ricinoleic acid | 7.26 ± 1.2 | 11.1 ± 0.80 |

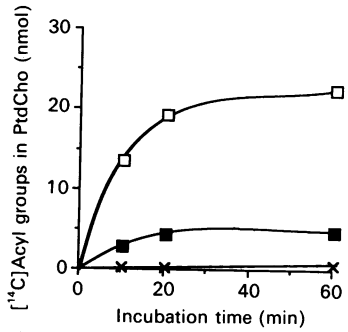



**Fig. 3. Incorporation of [$^{14}$C]oleate and [$^{14}$C]ricinoleate from [$^{14}$C]acyl-CoA in PtdCho by microsomal preparations from developing castor-bean endosperm**

Microsomal preparations (equivalent to 0.15 mg of microsomal protein) were incubated with 30 nmol of either [$^{14}$C]ricinoleoyl-CoA (■) or [$^{14}$C]oleoyl-CoA (□) (sp. radioactivity 1000 d.p.m./nmol) or a mixture of 30 nmol each of [$^{14}$C]ricinoleoyl-CoA and unlabelled oleoyl-CoA (×) in the presence of 2 mg of BSA.

**Table 3. Positional distribution of acyl groups and [$^{14}$C]acyl groups in PtdCho in microsomal preparations of developing castor-bean microsomes incubated with [$^{14}$C]oleoyl-CoA and NADH**

PtdCho from microsomal preparations (equivalent to 2.4 mg of microsomal protein) was isolated before and after incubation with [$^{14}$C]oleoyl-CoA (400 nmol; sp. radioactivity 4500 d.p.m./nmol), 12 $\mu$mol NADH and 40 mg of BSA in a total volume of 4 ml for 60 min. The PtdCho was treated with phospholipase $A_2$, and the acyl composition at positions sn-1 and sn-2 was determined as described in the Materials and methods section. The total incorporation of [$^{14}$C]acyl groups into PtdCho after incubation was 120 nmol

| | | Distribution of acyl groups (mol% in each position) | | | | | |
|---|---|---|---|---|---|---|---|
| | Position | Palmitate | Stearate | Oleate | Linoleate | Linolenate | Ricinoleate |
| Before incubation | *sn*-1 | 34.6 | 9.0 | 23.7 | 29.1 | 1.5 | 0.5 |
| | *sn*-2 | 2.3 | 1.0 | 38.7 | 44.1 | 4.5 | 8.9 |
| After incubation | *sn*-1 | 32.4 | 10.3 | 17.0 | 33.0 | 2.0 | 3.2 |
| | *sn*-2 | 3.6 | 1.6 | 21.0 | 49.4 | 3.9 | 20.0 |

| | | Distribution of [$^{14}$C]acyl groups (% of radioactivity in each position) | | |
|---|---|---|---|---|
| Position | Relative radioactivity (% of total radioactivity in both positions) | Oleate | Linoleate | Ricinoleate |
| *sn*-1 | 15.6 | 81.1 | 18.9 | 0 |
| *sn*-2 | 84.4 | 43.2 | 10.8 | 46.0 |

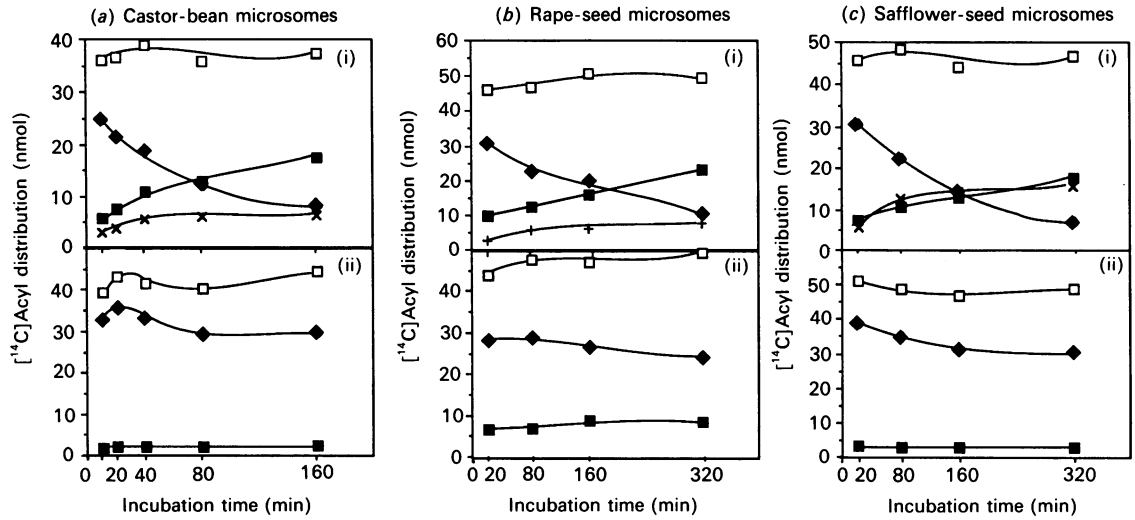



**Fig. 4. Metabolism by microsomal preparations of developing castor bean (*a*), oil-seed rape seed (*b*) and safflower seed (*c*) of [$^{14}$C]ricinoleoyl-PtdCho (i) and [$^{14}$C]oleoyl-PtdCho (ii) synthesized *in situ***

PtdCho was labelled with either [$^{14}$C]ricinoleate (i) or [$^{14}$C]oleate (ii) by incubating the microsomal preparations (equivalent to 0.6 mg of microsomal protein) with [$^{14}$C]ricinoleoyl-CoA or [$^{14}$C]oleoyl-CoA (40 nmol; sp. radioactivity 8000 d.p.m./nmol) respectively in the presence of lysoPtdOH (75 nmol). The radioactivity distribution in chloroform-soluble lipids was determined at various incubation times. Symbols: □, total chloroform-soluble lipids; ◆, PtdCho; ■, non-esterified fatty acids; +, diacylglycerol; ×, triacylglycerol.

existence of a phospholipase that rapidly releases the ricinoleic acid from PtdCho. The existence of such a phospholipase was investigated further. Microsomal membranes incubated with [$^{14}$C]oleoyl-CoA or [$^{14}$C]ricinoleoyl-CoA in the presence of supplied lyso-PtdCho became rapidly enriched with *sn*-2-[$^{14}$C]-oleoyl-PtdCho and *sn*-2-[$^{14}$C]ricinoleoyl-PtdCho respectively (Fig. 4*a*). After 10 min incubation 84% and 70% of the radioactivity in the chloroform-soluble lipids was found in [$^{14}$C]oleoyl-PtdCho and [$^{14}$C]ricinoleoyl-PtdCho respectively. No further significant increase occurred in radioactivity in the chloroform phase up to 160 min of incubation, indicating that essentially all the radioactive acyl-CoA substrate was utilized in the first 10 min of incubation. The further distribution of radioactivity in lipids with longer incubation times was investigated. In incubations with [$^{14}$C]ricinoleate the radioactivity in PtdCho decreased with an almost concomitant increase in the free [$^{14}$C]ricinoleic acid and, to a lesser extent, in the triacylglycerol (Fig. 4*a*, i). In similar incubations with [$^{14}$C]oleoyl-CoA the radioactivity in PtdCho exhibited only a slight decrease with incubation and with little increase in the free [$^{14}$C]oleic acid (Fig. 4*a*, ii).

The results therefore indicate that castor-bean endosperm has a phospholipase activity that exhibits acyl-specificity and is responsible for the release of ricinoleic acid from PtdCho. Is this phospholipase activity confined to ricinoleic acid-synthesizing species such as castor bean? To assess this possibility, similar experiments were carried out with microsomal preparations from other oil-seed species that do not synthesize ricinoleic acid, i.e. oil-seed rape and safflower. The results (Figs. 4*b* and 4*c*) show that both species, as in the case of castor bean, rapidly synthesized [$^{14}$C]ricinoleoyl-PtdCho and [$^{14}$C]oleoyl-PtdCho from [$^{14}$C]-ricinoleoyl-CoA and [$^{14}$C]oleoyl-CoA respectively in the presence of lyso-PtdCho. The distribution of radioactivity with further incubation showed similar patterns to that found in the castor-bean preparations. The only noteworthy difference among these three species was in the radioactivity lost from [$^{14}$C]ricinoleoyl-PtdCho and not found in free ricinoleic acid. In castor bean and safflower this radioactivity was recovered in triacylglycerol whereas in oil-seed rape it largely accumulated in diacylglycerol.

**Table 4. Effect of ATP, CoA and $Mg^{2+}$ on the distribution of [$^{14}$C]ricinoleate in lipids in microsomal preparations from developing castor-bean endosperm incubated with [$^{14}$C]oleoyl-CoA and NADH**

Microsomal preparations (equivalent to 0.6 mg of microsomal protein) were incubated with [$^{14}$C]oleoyl-CoA (100 nmol; sp. radioactivity 4500 d.p.m./nmol), NADH (3 μmol), BSA (20 mg) and ricinoleoyl-CoA (300 nmol) in the presence and in the absence of ATP, CoA and $MgCl_2$ (2, 1 and 10 μmol respectively) for 60 min. Results are the means ± S.E.M. for two incubations.

| | Acyl groups recovered (nmol) | |
|---|---|---|
| ATP, CoA and $Mg^{2+}$... | − | + |
| Ricinoleate in acyl-CoA | 123.6 ± 0.2 | 226.5 ± 3.2 |
| [$^{14}$C]Ricinoleate in acyl-CoA | 8.17 ± 1.45 | 18.42 ± 0.23 |
| [$^{14}$C]Acyl groups in chloroform-soluble lipids | 67.2 ± 0.88 | 63.3 ± 1.6 |
| [$^{14}$C]Ricinoleate in PtdCho | 11.38 ± 0.65 | 14.11 ± 0.28 |
| Free [$^{14}$C]Ricinoleic acid | 24.82 ± 0.51 | 9.95 ± 1.00 |

### Triacylglycerol assembly with ricinoleate

The utilization, in triacylglycerol biosynthesis, of the ricinoleic acid liberated from position *sn*-2 of *sn*-PtdCho by the acyl-specific phospholipase activity will require its activation to the CoA ester. Microsomal membranes were incubated with [$^{14}$C]oleoyl-CoA and NADH together with the necessary cofactors for an acyl-CoA synthetase (i.e. ATP, CoA and $Mg^{2+}$). Unlabelled ricinoleoyl-CoA was added also to the incubation mixture in order to trap the [$^{14}$C]ricinoleoyl-CoA synthesized. In the presence of cofactors for fatty acid activation the radioactivity recovered in [$^{14}$C]ricinoleoyl-CoA was over 2-fold that found in incubations minus cofactors (Table 4). The radioactivity in the free ricinoleic acid, on the other hand, decreased in the presence of fatty acid activation by a similar amount (Table 4). The

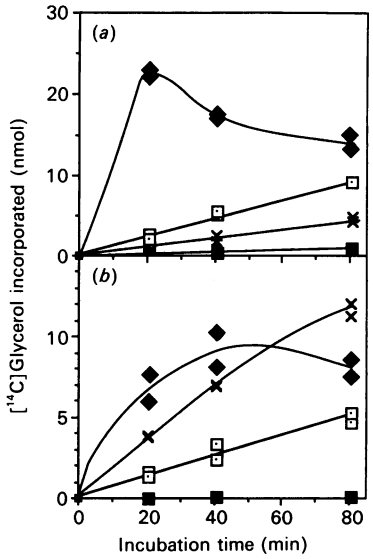



**Fig. 5. Incorporation of [$^{14}$C]Gro into lipids by microsomal preparations from developing castor-bean endosperm incubated with oleoyl-CoA (*a*) or ricinoleoyl-CoA (*b*) in the presence of [$^{14}$C]Gro3*P***

Microsomal preparations (equivalent to 0.3 mg of microsomal protein) were incubated with 200 nmol of either oleoyl-CoA (*a*) or ricinoleoyl-CoA (*b*) in the presence of *sn*-[$^{14}$C]Gro3*P* (1 $\mu$mol, sp. radioactivity 3000 d.p.m./nmol), BSA (10 mg) and $MgCl_2$ (10 $\mu$mol) for various periods of time. Symbols: ⊡, PtdCho; ◆, PtdOH; ■, diacylglycerol; ×, triacylglycerol.

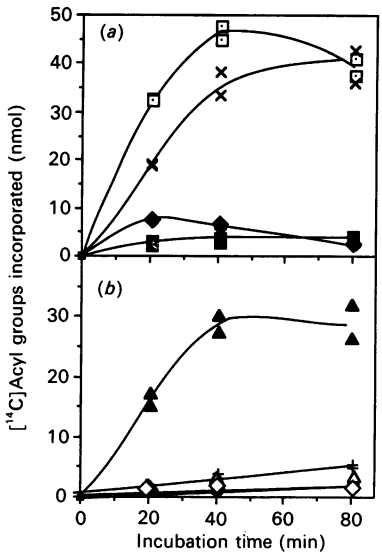



**Fig. 6. Incorporation of [$^{14}$C]oleate and [$^{14}$C]ricinoleate into lipids by microsomal preparations from developing castor-bean endosperm incubated with Gro3*P* and an equimolar mixture of [$^{14}$C]oleoyl-CoA and [$^{14}$C]ricinoleoyl-CoA**

Microsomal preparations (equivalent to 0.3 mg of microsomal protein) were incubated with [$^{14}$C]oleoyl-CoA and [$^{14}$C]ricinoleoyl-CoA (100 nmol of each, sp. radioactivity 3000 d.p.m./nmol) in the presence of Gro3*P* (1 $\mu$mol), BSA (10 mg) and $MgCl_2$ (10 $\mu$mol) for various periods of time. Symbols: ⊡, PtdCho; ◆, PtdOH; ■, diacylglycerol; ×, triacylglycerol; ◇, triacylglycerol with no ricinoleoyl groups; +, triacylglycerol with one ricinoleoyl group; ▲, triacylglycerol with two ricinoleoyl groups; △, triacylglycerol with three ricinoleoyl groups.

[$^{14}$C]ricinoleate recovered in PtdCho was similar for both incubations (Table 4).

Microsomal preparations incubated with [$^{14}$C]Gro3*P* and either ricinoleoyl-CoA or oleoyl-CoA efficiently acylated the glycerol backbone to yield radioactive PtdOH, PtdCho and triacylglycerol. In time-course studies (Fig. 5) with ricinoleoyl-CoA the rate of accumulation of $^{14}$C-labelled triacylglycerol was over twice that observed with oleoyl-CoA as acyl donor. In the presence of oleoyl-CoA, however, considerably more radioactive PtdOH and PtdCho accumulated. It should also be noted that with oleoyl-CoA a small, but significant, amount of radioactivity was associated with the diacylglycerol, whereas virtually no label was detected in this lipid with ricinoleoyl-CoA as the acyl donor. At 80 min of incubation and with oleoyl-CoA as the acyl donor, 87% of the radioactivity in triacylglycerol was present in molecular species lacking ricinoleate with the other 13% in species with one ricinoleoyl moiety only (results not shown). The radioactivity in the triacylglycerols synthesized with ricinoleoyl-CoA as acyl donor was, at 80 min incubation, distributed mainly in molecular species with three and two ricinoleoyl moieties (65% and 32% respectively) and with the remaining radioactivity in species with only one ricinoleoyl group (results not shown).

To investigate further the acylating properties of the glycerol-acylating activity observed in the castor-bean preparations, microsomal membranes were incubated with unlabelled Gro3*P* and a mixed [$^{14}$C]acyl-CoA substrate that consisted of equimolar [$^{14}$C]oleoyl-CoA and [$^{14}$C]ricinoleoyl-CoA. Under these conditions, PtdCho and triacylglycerol were rapidly labelled whereas relatively little radioactivity entered PtdOH and the diacylglycerol (Fig. 6*a*). At the most, only 30% of the radioactivity found in the PtdOH was, at all incubation times, in molecular species with hydroxylated acyl groups (results not shown). Also, only a minor part of the radioactivity in diacylglycerol was associated with species containing ricinoleate (results not shown). The $^{14}$C distribution in the different acyl groups of the PtdCho showed that up to 15% of the radioactivity was associated with [$^{14}$C]ricinoleate (results not shown). Most of the radioactivity found in the triacylglycerol was associated with molecular species with two ricinoleoyl groups (75%), and species with one ricinoleoyl moiety accumulated somewhat more activity than those with only ricinoleate or only oleate (Fig. 6*b*). It should be noted that analysis of the radioactive distribution of the acyl groups in the different molecular species of the triacylglycerols gave the same proportions of [$^{14}$C]oleate to [$^{14}$C]ricinoleate as the total ricinoleate to total non-hydroxylated fatty acid in the lipid species as deduced from their migration values on t.l.c. (see the Materials and methods section).

## DISCUSSION

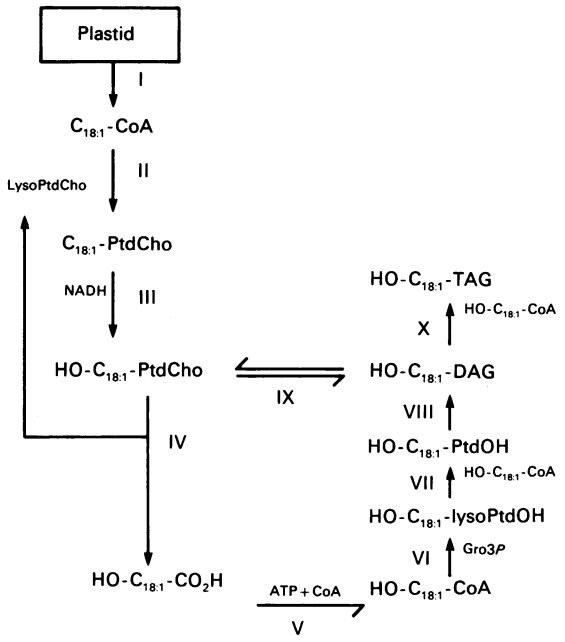
Microsomal membrane preparations from the developing endosperm of castor bean catalysed the transfer of oleate from [$^{14}$C]oleoyl-CoA to PtdCho. In the presence of NADH radioactive ricinoleate was synthesized from oleate, and this largely accumulated in neutral lipid, thus confirming previous observations (Moreau & Stumpf, 1981). Hydroxylase activity did not require $\alpha$-tocopherol as reported previously (Moreau & Stumpf, 1981). In the present investigation significant [$^{14}$C]ricinoleate was recovered in PtdCho, but most of the radioactive ricinoleate accumulated as free fatty acid. The addition of unlabelled ricinoleoyl-CoA to incubation mixtures of microsomes containing [$^{14}$C]oleoyl-CoA and NADH did not increase the low [$^{14}$C]ricinoleate concentration found in the acyl-CoA fraction nor decrease the [$^{14}$C]ricinoleate concentration in PtdCho and free fatty acid. Therefore [$^{14}$C]ricinoleoyl-CoA is not formed as a free intermediate by hydroxylase and thus it is not likely that the enzyme can utilize oleoyl-CoA as substrate.

The addition of NADH to microsomal membranes incubated with [$^{14}$C]oleoyl-CoA was not only necessary for the formation of [$^{14}$C]ricinoleate but also decreased the total radioactivity recovered in PtdCho with a corresponding amount of label appearing in free ricinoleic acid. These observations strongly indicate that the [$^{14}$C]oleate in oleoyl-PtdCho was the precursor of ricinoleic acid. Further evidence for this conclusion was that the [$^{14}$C]ricinoleoyl-PtdCho, formed by the acylation of added lyso-PtdCho with [$^{14}$C]ricinoleoyl-CoA, was specifically hydrolysed to yield free [$^{14}$C]ricinoleic acid. The presence of a ricinoleoyl-specific phospholipase A was not restricted to oil-seeds that accumulate ricinoleate and was also demonstrated in microsomal preparations from developing cotyledons of safflower and oil-seed rape. The apparent general occurrence of this phospholipase might therefore indicate its possible role in membrane repair mechanisms by specifically removing oxidized acyl groups from membrane lipids in order to preserve bilayer structure and function (see van Kuijk *et al.*, 1987). In the present study it was not possible to establish whether the phospholipase was positionally specific for the ricinoleate at position *sn*-2 of PtdCho. It was also evident that the ricinoleate can be made available from PtdCho by other routes than by the action of phospholipase $A_2$ and particularly in experiments with safflower microsomal preparations. Here a substantial part of the labelled ricinoleate was re-distributed, by unknown reactions, from PtdCho to the triacylglycerols. It has been shown previously that safflower microsomal preparation can form triacylglycerol with acyl groups from PtdCho in the apparent absence of acyl-CoA and particularly in microsomal membranes with elevated concentrations of diacylglycerol (Stobart & Stymne, 1985*a*).

Endogenous ricinoleate in microsomal PtdCho was almost entirely associated with position *sn*-2, where it amounted to 9% of the fatty acid in this position. This concentration probably reflects the steady state *in vivo* of ricinoleate synthesis and removal. In fact, the ricinoleate in PtdCho almost completely disappeared after incubating the microsomal membranes in buffer for 90 min at 25 °C (M. Bafor, M. A. Smith, L. Jonsson, K. Stobart & S. Stymne, unpublished work). The ricinoleate in positions *sn*-2 of *sn*-PtdCho increased to 20% in microsomal membranes incubated for 60 min with NADH and [$^{14}$C]oleoyl-CoA, whereas the amount in position *sn*-1 rose from a trace to only 3%, indicating that hydroxylation occurred mainly on position *sn*-2. The selectivity of the hydroxylase for the oleate in positions *sn*-2 of *sn*-PtdCho was further supported by the distribution of radioactive acyl groups in PtdCho, where substantial [$^{14}$C]ricinoleate resided in position *sn*-2 with none in position *sn*-1. In contrast, the [$^{14}$C]linoleate formed by desaturation of [$^{14}$C]oleoyl-PtdCho was present at both positions. Cell-free preparations from other developing oil-seeds such as safflower (Slack *et al.*, 1979; Stobart & Stymne, 1985*b*), borage (Griffiths *et al.*, 1988) and linseed (Stymne *et al.*, 1989) also desaturate the oleate at both sn-positions of sn-PtdCho.

Although ricinoleoyl-CoA was not the immediate product of the hydroxylation, it was formed from the free ricinoleic acid released from the PtdCho by an acyl-CoA synthetase enzyme present in the castor-bean microsomal preparation. Ricinoleoyl-CoA was an efficient acyl donor in the acylation of Gro3*P* to yield PtdOH and triacylglycerols. In incubations with microsomal membranes, [$^{14}$C]Gro3*P* and ricinoleoyl-CoA, the [$^{14}$C]glycerol also entered PtdCho but less efficiently than with oleoyl-CoA as substrate. The incorporation of glycerol backbone into PtdCho from the Gro3*P* pathways is, in oil-seeds, believed to be via the exchange of phosphocholine groups between a diacylglycerol pool and the PtdCho, rather than the net synthesis of PtdCho (Slack *et al.*, 1985; Stobart & Stymne, 1985*a*). It is therefore possible that the lower amount of [$^{14}$C]glycerol found in PtdCho with the ricinoleoyl-CoA substrate compared with the oleoyl-CoA could be attributed to a rapid utilization of ricinoleoyl-containing diacyglycerols in triacylglycerol synthesis, thus limiting the amount of ricinoleoyl-containing diacylglycerols available for interconversion with PtdCho. Although the incorporation of [$^{14}$C]glycerol from [$^{14}$C]Gro3*P* into triacylglycerol with ricinoleoyl-CoA as acyl donor was over twice that observed with oleoyl-CoA, the actual mass of triacylglycerol synthesized could be similar with both acyl-CoA substrates. In the incubations with the oleoyl-CoA the specific radioactivity of the diacylglycerol pool was probably diluted to a greater extent with non-radioactive glycerol backbone derived from interconversion with PtdCho.

When an equimolar mixture of [$^{14}$C]oleoyl-CoA and [$^{14}$C]-ricinoleoyl-CoA was supplied to the microsomal preparation in the presence of unlabelled Gro3*P*, 75% of the [$^{14}$C]triacylglycerol species synthesized had two [$^{14}$C]ricinoleoyl moieties and one [$^{14}$C]oleoyl moeity. Thus, the majority of the [$^{14}$C]diacylglycerols used for triacylglycerol assembly must have been ricinoleoyl-containing despite the fact that only a minor part of the [$^{14}$C]diacylglycerols that accumulated contained [$^{14}$C]ricinoleate.




**Scheme 1. Proposed pathway for the synthesis of ricinoleic acid and its incorporation into triacylglycerols in developing castor-bean (*Ricinus communis*) endosperm**

Step I. Oleic acid, synthesized in the plastid, is exported to the cytosol and activated to oleoyl-CoA. Step II. An acyl-CoA: lysoPtdCho acyltransferase transfers the oleate from oleoyl-CoA to lysoPtdCho to form oleoyl-PtdCho. Step III. The $\Delta^{12}$-fatty acid hydroxylase catalyses the conversion of oleoyl-PtdCho into ricinoleoyl-PtdCho at mainly position sn-2. Step IV. A ricinoleoyl-specific phospholipase A hydrolyses the PtdCho into free ricinoleic acid and lysoPtdCho. The lysoPtdCho can be re-acylated with oleate from oleoyl-CoA. Step V. Ricinoleic acid is activated to its CoA ester by an acyl-CoA synthetase. Step VI. Ricinoleate from ricinoleoyl-CoA is acylated to Gros3*P* to yield lysoPtdOH and (step VII) PtdOH. Step VIII. A phosphohydrolase converts the ricinoleoyl-PtdOH into a diacylglycerol (DAG): Step IX. Ricinoleoyl-containing diacylglycerols can enter, to some extent, PtdCho by a diacylglycerol-PtdCho interconversion. Step X. The diacylglycerol acyltransferase enzyme selectively utilizes ricinoleoyl-containing diacylglycerols in the formation of triacylglycerol (TAG), thereby limiting the pool size of ricinoleoyl-containing diacylglycerol available for interconversion with PtdCho.

This may therefore indicate a selective utilization of ricinoleoyl-containing diacylglycerols by the diacylglycerol acyltransferase enzyme. A similar preference for diacylglycerols that contain triacylglycerol-dedicated acyl groups was demonstrated also in the utilization of medium-chain diacylglycerols by the diacylglycerol acyltransferase in microsomal preparations from the developing seeds of *Cuphea lanceolata* (Bafor *et al.*, 1990). Triricinoleate is the main molecular species in castor-bean oil and yet this species accumulated at a very low rate *in vitro*. This may be because the ratio of oleoyl-CoA to ricinoleoyl-CoA (1:1) was much higher than occurs *in vivo*.

Oleoyl-CoA and ricinoleoyl-CoA were efficient acyl donors in the acylation of exogenously supplied lysoPtdCho, catalysed by the microsomal castor bean acyl-CoA:lysoPtdCho acyltransferase. In the acyl-exchange reaction, however, between acyl-CoA and position *sn*-2 of sn-PtdCho, i.e. incorporation of acyl groups from acyl-CoA into PtdCho in the absence of added lyso acceptor (Stymne & Stobart, 1984), the incorporation of ricinoleate from ricinoleoyl-CoA into PtdCho was negligible in the presence of competing oleoyl-CoA substrate. Therefore the [$^{14}$C]ricinoleate that entered PtdCho (10–15% of the total label in this lipid) in incubations with equimolar mixtures of [$^{14}$C]-oleoyl-CoA and [$^{14}$C]ricinoleoyl-CoA in the presence of Gro3*P* was most probably derived from the interconversion of [$^{14}$C]-ricinoleoyl-containing diacylglycerols and PtdCho. This would indicate that ricinoleoyl-containing diacylglycerol species were not totally selected against in the synthesis of PtdCho.

The biosynthesis of ricinoleic acid and its mode of incorporation into storage triacylglycerols in the developing castor-bean endosperm is summarized in Scheme 1.

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