Biosynthesis of Triacylglycerols Containing Ricinoleate in Castor Microsomes Using 1-Acyl-2-oleoyl-*sn*-glycero-3-phosphocholine as the Substrate of Oleoyl-12-hydroxylase

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ABSTRACT: We have examined the biosynthetic pathway of triacylglycerols containing ricinoleate to determine the steps in the pathway that lead to the high levels of ricinoleate incorporation in castor oil. The biosynthetic pathway was studied by analysis of products resulting from castor microsomal incubation of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine, the substrate of oleoyl-12-hydroxylase, using high-performance liquid chromatography, gas chromatography, mass spectrometry, and/or thin-layer chromatography. In addition to formation of the immediate and major metabolite, 1-palmitoyl-2-[^{14}C]ricinoleoyl-*sn*-glycero-3-phosphocholine, ^{14}C-labeled 2-linoleoyl-phosphatidylcholine (PC), and ^{14}C-labeled phosphatidylethanolamine were also identified as the metabolites. In addition, the four triacylglycerols that constitute castor oil, triricinolein, 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol, 1,2-diricinoleoyl-3-linoleoyl-*sn*-glycerol, 1,2-diricinoleoyl-3-linolenoyl-*sn*-glycerol, were also identified as labeled metabolites in the incubation along with labeled fatty acids: ricinoleate, oleate, and linoleate. The conversion of PC to free fatty acids by phospholipase A_2 strongly favored ricinoleate among the fatty acids on the *sn*-2 position of PC. A major metabolite, 1-palmitoyl-2-oleoyl-*sn*-glycerol, was identified as the phospholipase C hydrolyte of the substrate; however, its conversion to triacylglycerols was blocked. In the separate incubations of 2-[^{14}C]ricinoleoyl-PC and [^{14}C]ricinoleate plus CoA, the metabolites were free ricinoleate and the same triacylglycerols that result from incubation with 2-oleoyl-PC. Our results demonstrate the proposed pathway: 2-oleoyl-PC → 2-ricinoleoyl-PC → ricinoleate → triacylglycerols. The first two steps as well as the step of diacylglycerol acyltransferase show preference for producing ricinoleate and incorporating it in triacylglycerols over oleate and linoleate. Thus, the productions of these triacylglycerols in this relatively short incubation (30 min), as well as the availability of 2-oleoyl-PC *in vivo*, reflect the *in vivo* drive to produce triricinolein in castor bean.

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Ricinoleate has many industrial uses; however, the only commercial source of ricinoleate, castor (*Ricinus communis* L.) bean, contains the toxin ricin and potent allergens that pose serious health hazards to growers and processors. Because of the potential danger of these components in harvest and processing, it is desirable to produce ricinoleate in an oilseed of a transgenic plant lacking these components. The cDNA for oleoyl-12-hydroxylase, the key enzyme in the biosynthesis of ricinoleate, has recently been cloned (1). Expression of this enzyme in transgenic plants resulted in low levels of hydroxy fatty acids (2) compared to castor oil (20% vs. 90% ricinoleate in castor). It is therefore important to maximize expression of the hydroxylase and optimize incorporation of ricinoleate into triacylglycerols to develop transgenic plants that produce higher levels of ricinoleate. To accomplish this, it is essential to determine which enzymatic steps drive ricinoleate into triacylglycerols. It is also crucial to determine how the oleate supply is maintained for oleoyl-12-hydroxylase, since the final oil contains 5% or less of oleate.

In order to optimize incorporation of ricinoleate into triacylglycerols in a transgenic plant, the biosynthetic pathway of triricinolein must be known. Bafor *et al.* (3) proposed the biosynthetic pathway of triricinolein from microsomal incubations of oleoyl-CoA which is rapidly incorporated into 2-oleoyl-phosphatidylcholine (PC), the proposed substrate of oleoyl-12-hydroxylase (4). We report here the identifications of some intact metabolites of 2-oleoyl-PC, including the immediate metabolite of oleoyl-12-hydroxylase, 2-ricinoleoyl-PC, free ricinoleate and triacylglycerols containing rici-

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Abbreviations: DGDG, digalactosyl diacylglycerol; FAB, fast atom bombardment; FAME, fatty acid methyl esters; FFA, free fatty acids; GC, gas chromatography; HPLC, high-performance liquid chromatography; 2-linolenoyl-PC, 1-acyl-2-linolenoyl-*sn*-glycero-3-phosphocholine; 2-linoleoyl-PC, 1-acyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; MGDG, monogalactosyl diacylglycerol; MS, mass spectrometry or mass spectrum; NAPE, *N*-acyl-phosphatidylethanolamine; 2-oleoyl-PC, 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; 2-ricinoleoyl-PC, 1-acyl-2-ricinoleoyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography.

noleate, using high-performance liquid chromatography (HPLC) of intact lipids, demonstrating the major biosynthetic pathway to triricinolein. We also report the identification of enzymatic steps which drive ricinoleate into triacylglycerols. The key enzyme in the pathway, oleoyl-12-hydroxylase, has been characterized recently using both 2-oleoyl-PC (5) as immediate substrate and oleoyl-CoA (6) which is the acyl donor for biosynthesis of the substrate, 2-oleoyl-PC (3,4).

EXPERIMENTAL PROCEDURES

Microsomal incubation. Microsomes from castor bean were prepared as described previously (5). In order to keep the enzymes active after the microsomes were thawed for use in incubations, antipain-dihydrochloride (5 μg/mL; Boehringer Mannheim Corp., Indianapolis, IN) was added to the extraction buffer and microsome suspension buffer during microsomal preparation. The microsomal incubation mixture included in a total volume of 1 mL was: sodium phosphate (0.1 M, pH 6.3), NADH (0.5 mol), ATP (0.5 μmol), $MgCl_2$ (0.5 μmol), catalase (1000 units), and microsomal fraction of endosperm from immature castor bean (15 μL, 138 μg of protein). The radioactive substrate, 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (0.125 μCi, 2.16 nmol, 58.0 Ci/mol; DuPont NEN, Boston, MA) in 20 μL of ethanol was added last into a screw- capped tube followed by immediate mixing. The mixture was then incubated in a shaking water bath for 30 min at 22°C. The incubation was stopped by addition of 3.75 mL of chloroform/methanol (1:2, vol/vol). The mixture was again mixed with 0.63 mL of chloroform and 0.63 mL of water. The lower chloroform layer containing the lipid extract was dried and fractionated on a silica HPLC system for the separation of lipid classes described here. The radioactive 2-ricinoleoyl-PC was prepared by a microsomal incubation of 2-[1-^{14}C]oleoyl-PC up to 48 times the incubation mixture given above (48 × volume). Then the PC fraction obtained was purified by the C_8 HPLC system described here to obtain radioactive 2-ricinoleoyl-PC. In the study of metabolism of free ricinoleate, radioactive ricinoleate was added to the incubation (1 h) in place of radioactive 2-oleoyl-PC as described here, and CoA (0.5 μmol) also was added to the incubation. Radioactive ricinoleate was prepared by the castor microsomal incubation of radioactive oleoyl-CoA (6), hydrolysis by sodium hydroxide, and purification by C_{18} HPLC system of free fatty acids (FFA) (7). For the purpose of identifying the fatty acid constituents of intact lipids, lipid extracts were hydrolyzed and methylated in 5% HCl/methanol (1 mL) at 80°C for 1 h. The fatty acid methyl esters (FAME) formed were extracted with 2 × 1 mL of hexane.

HPLC. Different HPLC systems were carried out as described previously (5). Radioactive lipids were separated by HPLC and identified by cochromatography with lipid standards. The flow rates of eluents of different HPLC systems were 1 mL/min. Different HPLC systems provided separations for various products as follows:

(i) Separation of lipid classes: Lipid classes were separated according to Singleton and Stikeleather (8) by a silica column (25 × 0.46 cm, 5 μm, Spherisorb S5W; Phase Separations, Norwalk, CT) with a linear gradient starting at isopropanol/hexane (4:3, vol/vol) to isopropanol/hexane/water (4:3:0.75, by vol) in 20 min, then isocratically for 15 min. A Pre-Sat silica saturation column (25 × 0.46 cm; Alltech Associates Inc., Deerfield, IL) was installed between the pump and injector to saturate the mobile phase with silica before it reached the analytical column. Free phosphatidylglycerol (PG), cardiolipin, phosphatidylinositol (PI), and phosphatidic acid standards were obtained by acidification of their salts (Sigma Chemical Co., St. Louis, MO) with HCl.

(ii) Separation of PC molecular species: PC molecular species were separated by a C_8 column (25 × 0.46 cm, 5 μm, Ultrasphere C8; Beckman Instruments Inc., Fullerton, CA) with a linear gradient of 90–100% methanol (containing 0.1% of conc. NH_4OH) in 40 min. A Pre-Sat silica saturation column was installed between the pump and injector to prevent the rapid deterioration of the C_8 column by the pH 9.5 mobile phase.

(iii) Separation of FAME: FAME were separated in the same manner as we previously described (5) using a short C_{18} column (5 × 0.46 cm, 3 μm, Microsorb MV; Rainin Instrument Co. Inc., Woburn, MA) to save time. A linear gradient of 90–100% methanol in 15 min was used for 10 min. For the purpose of identifying the FAME, a regular C_{18} column (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman) was used (7) with a linear gradient of 90–100% methanol in 40 min.

(iv) Separation of FFA: FFA were separated as we reported previously (7) using a C_{18} column (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman) with a linear gradient of 85–100% methanol (containing 0.05% HAc as ion suppressor) in 40 min.

(v) Separation of acylglycerol molecular species: Acylglycerol molecular species (triacylglycerols and diacylglycerols) were separated by the use of a C_{18} column (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman) with a linear gradient starting at 100% methanol to 100% isopropanol in 40 min as we have reported (9). Another linear gradient (9) starting at 100% methanol to methanol/isopropanol (50:50) in 40 min was used for the identification of 1,2-dioleoyl-*sn*-glycerol.

Mass spectrometry (MS) by fast atom bombardment (FAB). Lipids were applied to the probe of a Jeol HX-110 double-focusing instrument (Tokyo, Japan). Glycerol was added to the sample to create optimal conditions for FAB ionization. FAB ionization was performed using xenon gas at 6 keV. Ions were accelerated out of the ion source at 10 kV. Both positive and negative ion acquisitions were obtained with the exception of only negative-ion acquisitions obtained for PI since this compound gave no signal in the positive mode. Mass ranges scanned were 400–1300 daltons for positive-ion and 200–1300 daltons in the negative-ion mode. Scan speed was 5 s per decade. Ion source temperature was maintained at 37°C.

Identification of FAME by gas chromatography (GC) and GC–MS. All FAME were tentatively identified and quantitated with an HP6890 gas chromatograph (Hewlett-Packard Co., San Fernando, CA) and identities were confirmed by GC–MS using an HP5970A quadrupole-based mass selective detector as previously described (10). Data were analyzed

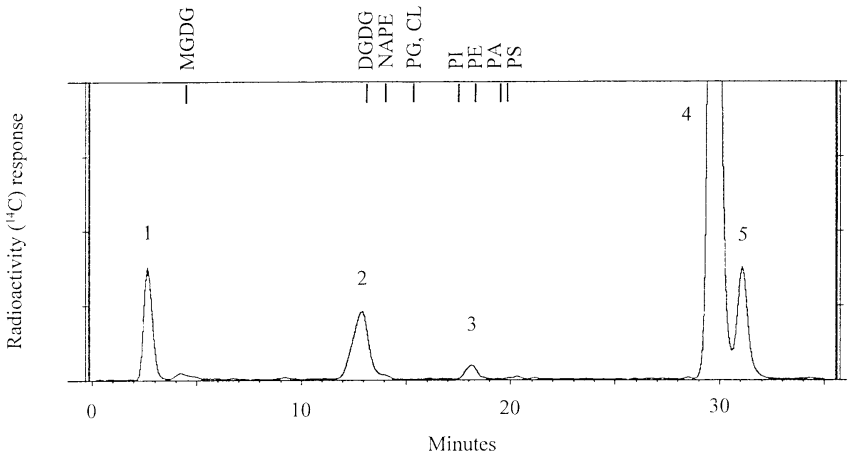


FIG. 1. Separation of lipid classes of total lipid extract from the castor microsomal incubation with 2-[^{14}C]oleoyl-phosphatidylcholine (PC) using a silica high-performance liquid chromatography (HPLC) system (see the Experimental Procedures section, HPLC, i). The radioactive peaks were: (1) acylglycerols and free fatty acids (FFA), retention time 2.7 min; (2) unknown, 13.0 min; (3) phosphatidylethanolamine (PE), 18.2 min; (4) PC, 29.8 min; (5) 2-ricinoleoyl-PC, 31.1 min. Retention times of other lipid classes in this HPLC system are also shown in the figure. MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; NAPE, *N*-acyl-phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine.

using Hewlett-Packard MS ChemStation (DOS series) software (HP G1034C).

Thin-layer chromatography (TLC) of phospholipids and glycolipids. Phospholipids were separated by TLC on silica gel plates which were first immersed in 100 mM ammonium sulfate and dried for 1.5 h at 120°C. Samples were applied to the plates in a spotting chamber. Separation was with the solvent system 1-propanol/chloroform/propanoic acid/0.1%(wt/vol) KCl (3:2:2:1, by vol). Glycolipids were separated on a silica gel plate in the solvent system chloroform/methanol/conc. ammonia (13:5:1, by vol). Bands containing lipids to be analyzed were routinely localized by iodine staining. Lipids were identified by co-migration with standards and by specific spray reagents (11). The radioactivity was localized by autoradiography with Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY).

RESULTS AND DISCUSSION

It is generally accepted that oleoyl-12-hydroxylase catalyzes the reaction from 2-oleoyl-PC to 2-ricinoleoyl-PC in castor bean. We have previously (5) used the putative substrate, 2-oleoyl-PC, to characterize oleoyl-12-hydroxylase in microsomes from the endosperm of immature castor bean. In the previous study (5), the enzyme activity of oleoyl-12-hydroxylase was determined by the radioactivity of total ricinoleate after methanolysis of total lipid in the incubation. In this report, the intact lipid metabolites of 2-[^{14}C]oleoyl-PC have been identified, and the incorporation of radiolabel in each metabolite has been quantified in order to follow the label through the biosynthetic pathway of triacylglycerols containing ricinoleate.

The castor microsomal incubation of 2-[^{14}C]oleoyl-PC was performed as previously described (5). Figure 1 shows the radiochromatogram of the separated lipid classes from the total lipid extract of the incubation. By using standards, the radioactive peaks in Figure 1 were identified as follows: peak 1: acylglycerols (neutral lipids) and FFA, 2.7 min (retention time), 6% of total radioactivity; peak 2: unknown, 13.0 min, 7%; peak 3: phosphatidylethanolamine (PE), 18.2 min, 1%; peak 4: PC, 29.8 min, 78%; peak 5: 2-ricinoleoyl-PC, 31.1 min, 8%. Many standards of lipid classes were used for identifications as shown in Figure 1. Radioactive peak 2 in Figure 1 corresponded to digalactosyl diacylglycerol (DGDG) or *N*-acyl-PE (NAPE) on this HPLC system; however, it did not correspond to DGDG or NAPE on TLC. DGDG is synthesized from diacylglycerols by galactosylacyltransferases located in the plastid envelope (12), and NAPE is synthesized from PE and FFA by microsomal NAPE synthase (13). We have not yet identified the radioactive peak 2 in Figure 1.

Radioactive peak 3 in Figure 1 was identified as PE (tentatively) by HPLC retention time and TLC. However, the MS of this fraction [17.0–19.0 min in Fig. 1 which included PE and phosphatidylinositol (PI)] indicated the presence of PI. The FAB(–) showed the molecular ions, M – H (834 – 1 and 836 – 1), with their relative abundances at *m/z* 833 (52%) and 835 (27%) which correspond to 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoinositol and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoinositol, respectively. Other ions shown were *m/z* 671 (11%, M – 163 inositol), 571 (18%, M – 263 linoleoyl), 299 (40%, M – palmitoyl – linoleoyl), 281 (35%, oleoyl), 279 (60%, linoleoyl), 255 (100%, palmitoyl). The MS of peak 3 did not indicate the presence of PE. PE and PI were well separated by TLC as ammonium salts [with their

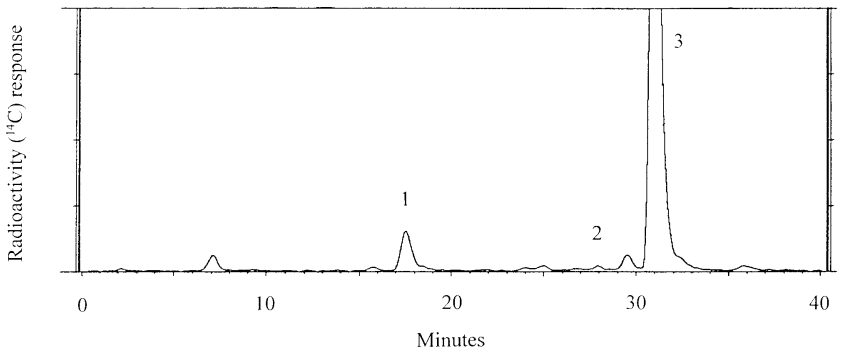


FIG. 2. Purification of 2-[^{14}C]ricinoleoyl-PC (peak 1), using a C_8 HPLC system (see the Experimental Procedures section, HPLC, ii). Radioactive peaks were: (1) 1-palmitoyl-2-ricinoleoyl-PC, 17.6 min; (2) 1-palmitoyl-2-linoleoyl-PC, 28.0 min; (3) 1-palmitoyl-2-oleoyl-PC, 31.0 min. See Figure 1 for abbreviations.

retention factor (R_f) values of 0.47 and 0.23, respectively] while HPLC did not resolve the free acids well. PE was radioactive on TLC, and its amount was not high enough to be detected by MS, while PI was not radioactive on TLC, and could be detected by MS. The radioactive peak 3 in Figure 1 thus contained radioactive PE together with unlabeled PI. Thus, the labeled 2-oleoyl-PC was metabolized in part to PE, but not to PI. The labeled PE could be derived from labeled diacylglycerols which were abundant in this incubation (as shown in Scheme 1) by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase in castor microsomes (14). The labeled PE also could be synthesized in castor microsomes by a base group exchange reaction (15). The biosynthesis of radioactive PI in this incubation was unlikely (16).

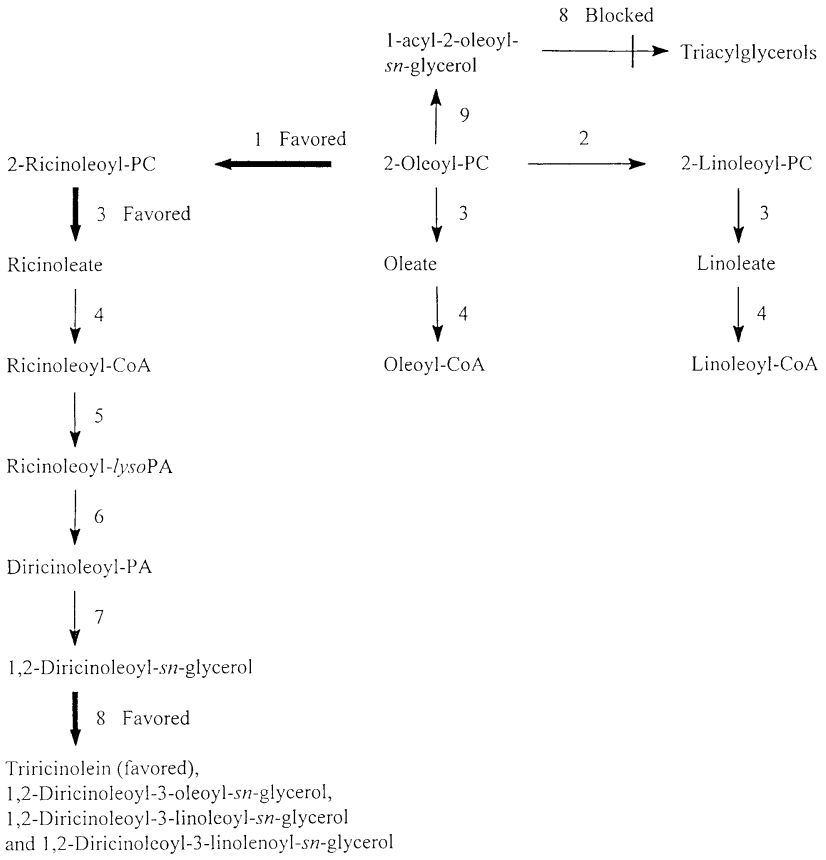
Since there was no 2-ricinoleoyl-PC standard available to us, peak 5 in Figure 1 was identified as follows. Half-minute fractions of radioactive peaks 4 and 5 in Figure 1 were collected and then hydrolyzed and methylated. HPLC of the radioactive FAME obtained showed that the radioactive peaks 4 and 5 on Figure 1 corresponded to 2-oleoyl-PC and 2-ricinoleoyl-PC, respectively. About 1% of peak 4 corresponded to radioactive 2-linoleoyl-PC. The radioactive peak 5, 2-ricinoleoyl-PC, was further purified by C_8 HPLC as shown in Figure 2. The radioactive peak 1 (19.8 min) in Figure 2 appeared to be 1-palmitoyl-2-ricinoleoyl-*sn*-glycero-3-phosphocholine by MS. A molecular weight of 776 was shown in MS of FAB(+). The MS of FAB(−) showed the characteristic negative ion of PC, 761 ($M - CH_3^-$). The hydrolysis of peak 1 in Figure 2 by phospholipase A_2 (from *Naja mocambique mocambique,* Sigma P-4034) showed the presence of free [^{14}C]ricinoleate by HPLC in the incubation product. We have thus identified 1-palmitoyl-2-[^{14}C]ricinoleoyl-*sn*-glycero-3-phosphocholine as the metabolite of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine by oleoyl-12-hydroxylase in castor microsomes. Since we have previously used 2-oleoyl-PC as the substrate of oleoyl-12-hydroxylase in the castor microsomal incubation (5) and we now identify its immediate metabolite, we have thus proved that 1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine is the substrate of oleoyl-12-hydroxylase in castor bean. Ricinoleate was mainly or completely made at the *sn*-2 position (3).

The only metabolites (fatty acids) of oleate from the *sn*-2 position of 2-oleoyl-PC were identified previously as ricinoleate and linoleate (5). Radioactive lipid classes shown in Figure 1 were hydrolyzed and methylated, and the FAME formed were separated by HPLC on a 5-cm, C_{18} column which resolves the three labeled FAME (5). All lipid classes contained radioactive ricinoleate, linoleate, and oleate. The distribution of labeled fatty acids in each lipid class is shown in Table 1. The PC fraction shown in Table 1 was the combination of radioactive peaks 4 and 5 in Figure 1 which included 2-oleoyl-PC (the substrate), 2-ricinoleoyl-PC, and 2-linoleoyl-PC. 2-Ricinoleoyl-PC was the major immediate metabolite of 2-oleoyl-PC. In the microsomal incubation, the conversion of PC to acylglycerols (neutral lipids) and FFA strongly favored ricinoleate among the three fatty acids on the *sn*-2 position of PC as shown by others (17). The incorporation of radioactivity into PE also showed preference for ricinoleate. Thus, the biosynthesis of PE likely occurred from the radioactive 1,2-diacyl-*sn*-glycerols shown in Scheme 1 by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (14). Since 1,2-diacyl-*sn*-glycerols were enriched

TABLE 1
Radioactive Fatty Acid Composition of Lipid Classes and Acylglycerols from the Castor Microsomal Incubation of 2-[^{14}C]Oleoyl-PC[a]

Lipid classes and acylglycerols	Ricinoleate	Linoleate	Oleate
PC	6	3	91
PE	52	4	44
Unknown	6	4	90
Acylglycerols and FFA	63	7	30
FFA	73	5	22
Triricinolein	100	0	0
Diricinoleoyl-linolenoyl-glycerol	100	0	0
Diricinoleoyl-linoleoyl-glycerol	96	4	0
Diricinoleoyl-oleoyl-glycerol	93	0	7
1-Palmitoyl-2-oleoyl-*sn*-glycerol	0	0	100

[a]PC, phosphatidylcholine; PE, phosphatidylethanolamine; FFA, free fatty acid.



SCHEME 1

in ricinoleate, they were mostly formed after the enzymatic action of oleoyl-12-hydroxylase and phospholipase A_2 which provide ricinoleate for 1,2-diacyl-*sn*-glycerol. 1,2-Diacyl-*sn*-glycerols can also be formed by phospholipase C hydrolysis of the three labeled PC shown in Scheme 1, but this reaction is unlikely since 1-palmitoyl-2-oleoyl-*sn*-glycerol was identified as the major labeled metabolite (Fig. 5) while 1-palmitoyl-2-ricinoleoyl-*sn*-glycerol and 1-palmitoyl-2-linoleoyl-*sn*-glycerol were not identified. The biosynthesis of PE was unlikely from the base exchange reaction (15), incorporating free ethanolamine into PE, because the base exchange reaction would likely reflect the fatty acid composition of PC and thus not favor ricinoleate. The incorporation of label in the unknown peak 2 (Fig. 1) showed no fatty acid preference. The unknown peak 2 (Fig. 1) contained the unchanged diacylglycerol portion of PC which might be derived from PC by phospholipase C or D (18).

Radioactive FFA in the fraction of acylglycerols and FFA in Figure 1 (peak 1, 2–5 min) were identified by the C_{18} HPLC system for the separation of FFA as shown in Figure 3. Free ricinoleate, linoleate, and oleate were identified by HPLC cochromatography with the standards. The FFA represented about 23% of the radioactivity in this fraction, and the percentage radioactivity of each FFA shows that phospholipase A_2 favored release of ricinoleate (Table 1). Although the release of oleate was not favored in this study, it was much higher than that of previous studies (3,17) which showed that ricinoleate was specifically released from PC in castor microsomes.

The fraction of acylglycerols and FFA in Figure 1 (peak 1, 2–5 min) was cochromatographed with castor oil by the C_{18} HPLC system to separate molecular species of acylglycerols as shown in Figure 4. The radioactive triacylglycerols (peaks 1–4 in Fig. 4C) identified were the four major triacylglycerols in castor oil shown in Figure 4A, triricinolein (peak 1, the main triacylglycerols in castor oil, 8.5% of the total radioactivity in the fraction of acylglycerols and FFA), diricinoleoyl-linolenoyl-glycerol (peak 2, 0.8%), diricinoleoyl-linoleoyl-glycerol (peak 3, 4.0%), and diricinoleoyl-oleoyl-glycerol (peak 4, 11.9%). The four major triacylglycerols in castor oil (peaks 1–4, Fig. 4A) were identified by GC and GC–MS of the FAME after methanolysis of these peaks. The radioactive peak 5 in Figure 4C at the retention time of 2.9 min corresponded to free ricinoleate and was about 16% of the total radioactivity in the fraction of acylglycerols and FFA. The radioactive peaks of free linoleate (3.5 min) and oleate (4.5 min) were not detectable in Figure 4C, and were masked by other radioactive peaks as shown in Figure 4C.

Radioactive peak 6 in Figure 4C with retention time 17.3 min, the largest radioactive peak representing 23% of the total radioactivity in the fraction of acylglycerols and FFA (peak 1, Fig. 1), did not correspond to any acylglycerols and FFA detectable in castor oil (Fig. 4A) and castor microsomes (Fig.

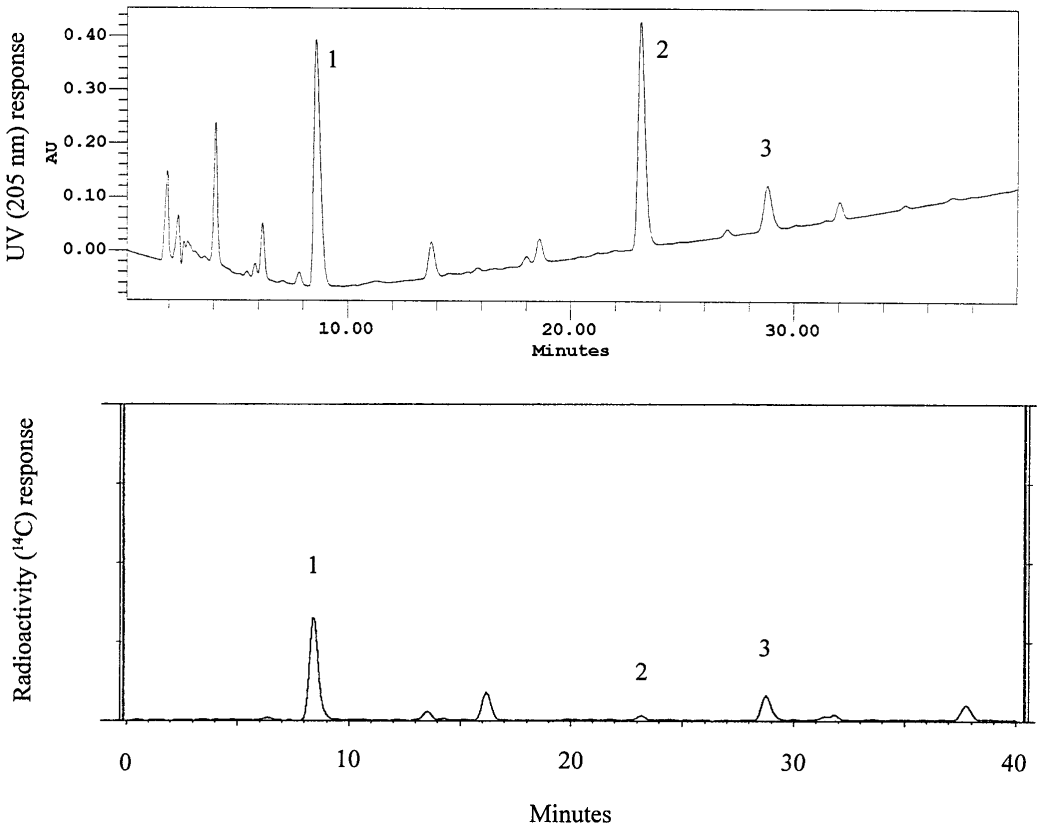


FIG. 3. HPLC identification of radioactive FFA in the fraction of acylglycerols and FFA (peak 1, Fig. 1) from the castor microsomal incubation of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine, using a C_{18} HPLC system (see the Experimental Procedures section, HPLC, iv). The sample was cochromatographed with free ricinoleate (10 μg), linoleate (10 μg), and oleate (5 μg). The radioactive peaks were: (1) ricinoleate, 8.57 min ; (2) linoleate, 23.12 min; (3) oleate, 28.82 min. UV, ultraviolet. See Figure 1 for other abbreviations.

4B). The HPLC of the FAME of peak 6 in Figure 4C after methanolysis showed that the only radioactive fatty acid was oleate. However, the GC showed equal amounts of palmitate and oleate only. The radioactive peak 6 in Figure 4C was coeluted with 1,2-dioleoyl-*sn*-glycerol as shown in Figure 5 using the linear gradient of 100% methanol to methanol/isopropanol (50:50) in 40 min. We have recently reported the HPLC of 45 synthetic diacylglycerols and triacylglycerols (9). The relative retention times of 1,3-dioleoyl-glycerol and 1,2-dioleoyl-*sn*-glycerol were 18.67 and 18.95 min, respectively, and they can be resolved by this HPLC system. The relative retention time of 1,2-dipalmitoyl-*sn*-glycerol was 18.97 min and was almost exactly the same as that of 1,2-dioleoyl-*sn*-glycerol. Even though we do not have the standard of 1-palmitoyl-2-oleoyl-*sn*-glycerol, it should be coeluted with 1,2-dioleoyl-*sn*-glycerol; and the radioactive peak 6 of Figure 4 was identified as 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol, the product of substrate hydrolysis by phospholipase C in castor microsomes. This labeled product also coeluted with 1,2-dioleoyl-*sn*-glycerol in the HPLC system of Figure 5. We can thus infer that phospholipase C activity toward PC is present in castor bean. The neutral metabolites (triacylglycerol) of 1-palmitoyl-2-oleoyl-*sn*-glycerol which eluted after 1-palmitoyl-2-oleoyl-*sn*-glycerol were minor, if any, as shown in Figure 4C. The minor radioactive peaks in Figure 4C were not identified. The unidentified radioactive peaks in Figure 4C might be triacylglycerols and diacylglycerols containing labeled ricinoleate, oleate, and/or linoleate.

Increasingly longer microsomal incubations with 2-[1-^{14}C]oleoyl-PC, up to 2 h, showed as in Figure 6 continuous increases in the labeling of acylglycerols and FFA (peak 1 of Fig. 1, including triricinolein), of the unknown (peak 2 of Fig. 1), and of PE (peak 3 of Fig. 1). However, radioactive 2-ricinoleoyl-PC (peak 5 of Fig. 1) was maximal at 30 min. Clearly, 2-ricinoleoyl-PC is an intermediate in the metabolic pathway. To further corroborate this, microsomal incubation of the radioactive 2-ricinoleoyl-PC showed the labeled metabolites, free ricinoleate, triricinolein, 1,2-diricinoleoyl-3-linoleoyl-*sn*-glycerol, and 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol as shown in Figure 7. Radioactive peak 6 in Figure 7 corresponded to 1-palmitoyl-2-oleoyl-*sn*-glycerol which was the same as peak 6 in Figure 4. Its origin needs to be investigated. 1-Palmitoyl-2-oleoyl-*sn*-glycerol was the hydrolyte of 2-oleoyl-PC by phospholipase C in the incubation.

Incubation with radioactive free ricinoleate also showed the incorporation of ricinoleate into triacylglycerols. The HPLC

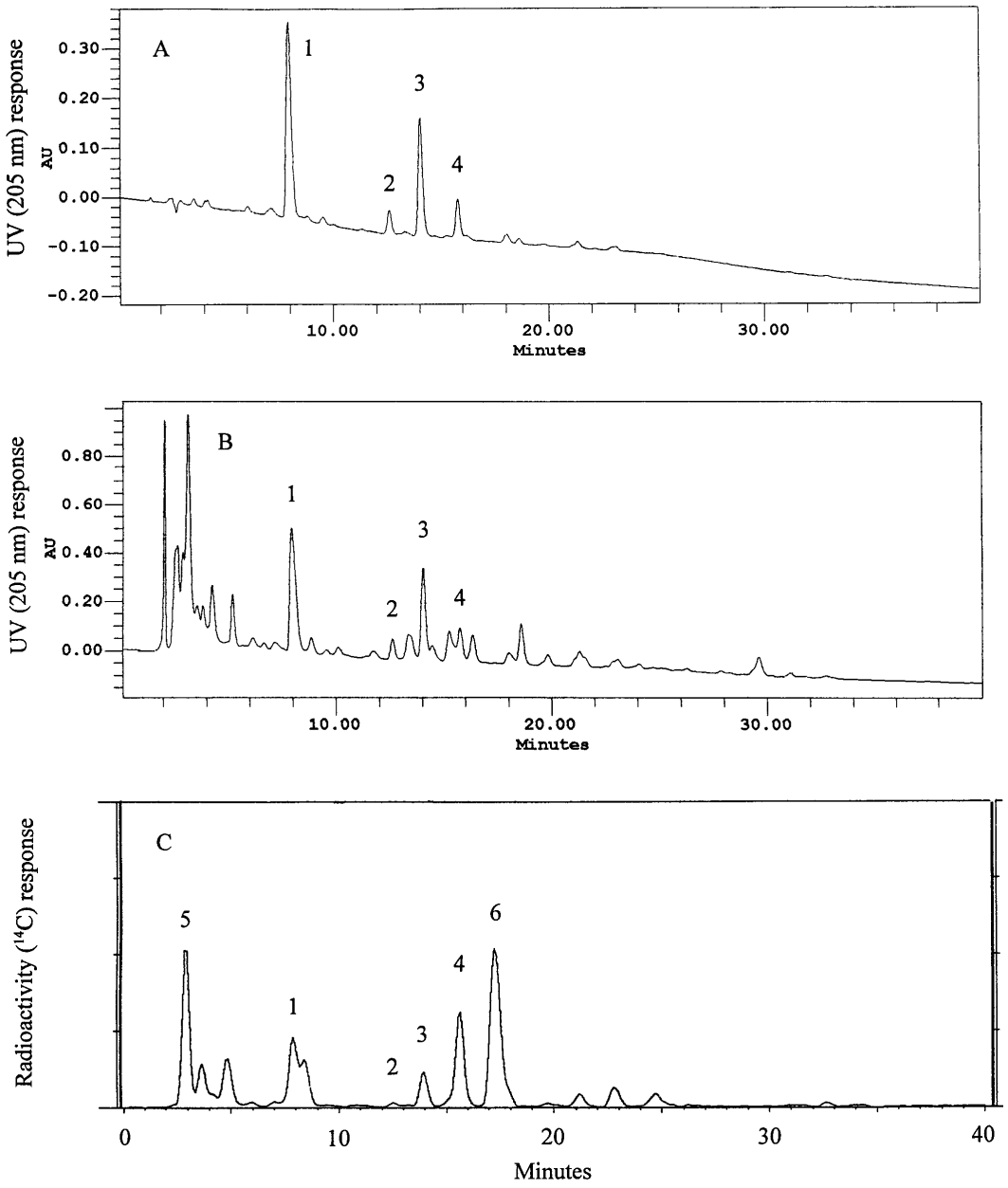


FIG. 4. HPLC identification of radioactive triacylglycerols in the fraction of acylglycerols and FFA (peak 1, Fig. 1) from the castor microsomal incubation of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine, using a C_{18} HPLC system (see the Experimental Procedures section, HPLC, v). A linear gradient from 100% methanol to 100% isopropanol in 40 min was used. Peak 1, triricinolein; peak 2, diricinoleoyl-linolenoyl-glycerol; peak 3, diricinoleoyl-linoleoyl-glycerol; peak 4, diricinoleoyl-oleoyl-glycerol. Figure 4A was the HPLC profile of castor oil (80 µg) which shows the retention times of triacylglycerols as: (peak 1) 7.94 min, (2) 12.62 min, (3) 14.05 min, (4) 15.79 min. Figure 4B was the HPLC profile of the fraction of acylglycerols and FFA of the lipid extract from the incubation (peak 1, Fig. 1) which was cochromatographed with castor oil (80 µg). The retention times were: (peak 1) 7.95 min, (2) 12.61 min, (3) 14.04 min, (4) 15.75 min. Figure 4C was the radiochromatogram of the same HPLC run as Figure 4B. The retention times obtained from the radioactive flow detector were: (peak 1) 7.9 min, (2) 12.6 min, (3) 14.0 min, (4) 15.7 min. Peak 5, free ricinoleate (2.9 min); peak 6, 1-palmitoyl-2-oleoyl-sn-glycerol (17.3 min). See Figures 1 and 3 for abbreviations.

of the fraction of acylglycerols and FFA cochromatographed with castor oil (Fig. 8) showed the identifications of radioactive triricinolein, 1,2-diricinoleoyl-3-linolenoyl-*sn*-glycerol, 1,2-diricinoleoyl-3-linoleoyl-*sn*-glycerol, and 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol. However, radioactive peak 6 of Figure 4C (1-palmitoyl-2-oleoyl-*sn*-glycerol, hydrolyte of the substrate 2-oleoyl-PC by phospholipase C) was not present in Figure 8 as expected. No incorporation of free ricinoleate into triacylglycerols was observed in the incubation without the addition of CoA. It is interesting that the incorporation of exoge-

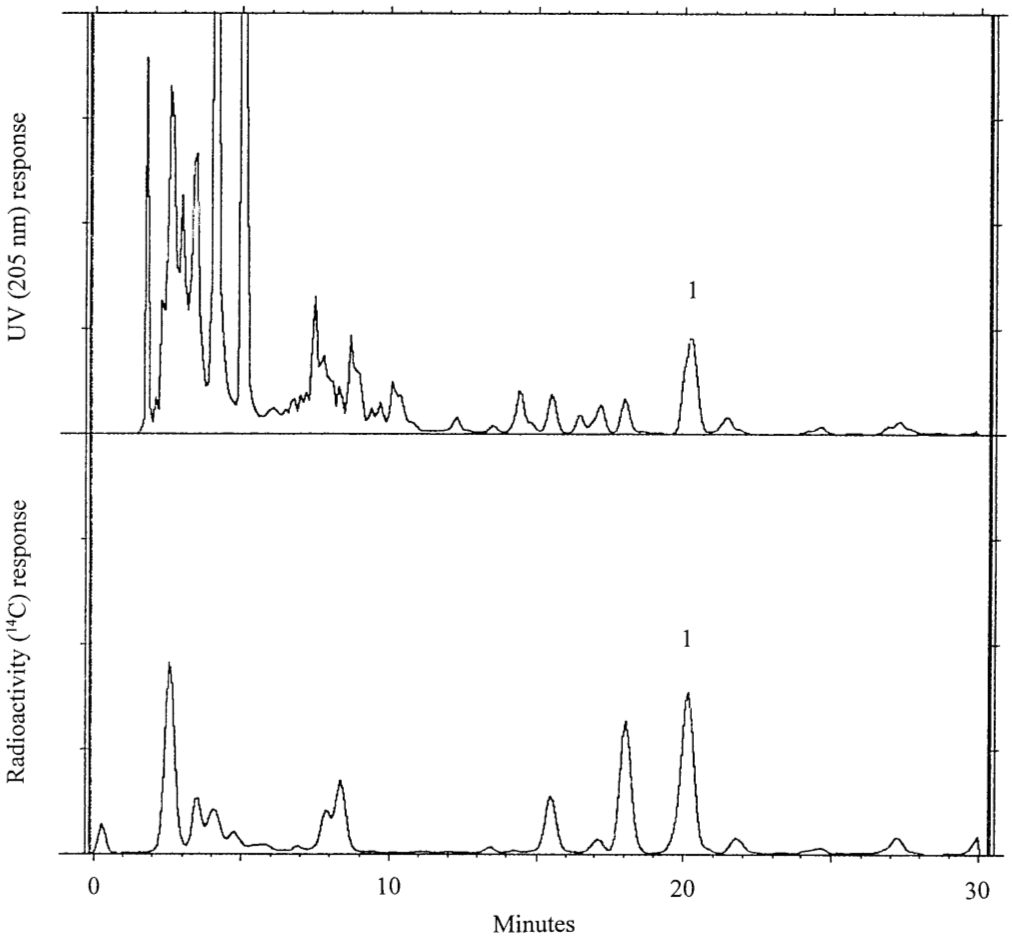


FIG. 5. HPLC identification of radioactive 1-palmitoyl-2-oleoyl-*sn*-glycerol in the fraction of acylglycerols and FFA (peak 1, Fig. 1) from the castor microsomal incubation of 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine, using a C_{18} HPLC system (see the Experimental Procedures section, HPLC, v). The linear gradient was 100% methanol to methanol/isopropanol (50:50) in 40 min. The sample was cochromatographed with 1,2-dioleoyl-*sn*-glycerol (50 μg), and its retention time (20.2 min, peak 1 of UV chromatogram) was the same (20.2 min) as that of peak 1 of the radiochromatogram. Both the UV chromatogram and radiochromatogram were from the printer of radioactivity flow detector. See Figures 1 and 3 for abbreviations.

nous free ricinoleate into triacylglycerols in the incubation required CoA while the incorporation of ricinoleate from 2-ricinoleoyl-PC or 2-oleoyl-PC into triacylglycerols does not. It is possible that lipids derived from these substrates participate in acyl exchange reactions as recently described (19). Endogenous CoA was available for the activation of ricinoleate formed from 2-ricinoleoyl-PC (Scheme 1) while it was not available for exogenous ricinoleate. We have previously added CoA to the incubation (5), and it has not made a difference in the total ricinoleate-containing lipids produced. The radioactivity ratio of peaks 2, 3, and 4 in Figure 8 was similar to the ratio of peaks 2, 3, and 4 of castor oil of Figure 4A. The ratio probably reflected the substrate (acyl CoA) specificity of the diacylglycerol acyltransferase (step 8, Scheme 1) and/or the availabilities of acyl CoA in castor endosperm *in vivo*. The radioactivity of triricinolein (peak 1, Fig. 8) was lower than those of peaks 3 and 4 in Figure 8 and was probably due to the depletion of ricinoleoyl CoA used in the biosynthesis of 1,2-diricinoleoyl-*sn*-glycerol in the incubation. The radioactive peak 6 in Figure 8 has not been identified yet and might be 1,2-diricinoleoyl-*sn*-glycerol, the intermediate in the pathway (Scheme 1), according to the elution characteristic of acylglycerols (9). The possible labeled 2-ricinoleoyl-PC (identical relative retention time) was also shown in the total lipid extract in the radiochromatogram (not shown) using the HPLC system of Figure 1. The incorporation of [^{14}C]ricinoleoyl-CoA into triacylglycerols in castor microsomes previously has been reported (3,20). The incorporation of the ammonium salt of [^{14}C]ricinoleate into triricinolein, diricinoleoyl-acyl-glycerols, ricinoleoyl-diacyl-glycerols, and ricinoleoyl-acyl-glycerols has also been recently reported in castor endosperm *in vivo* (20). We have shown the phospholipase A_2 hydrolysis of 2-ricinoleoyl-PC and the incorporation of ricinoleate from 2-ricinoleoyl-PC into triacylglycerols in castor microsomes, thus demonstrating the pathway *in vitro:* 2-oleoyl-PC → 2-ricinoleoyl-PC → ricinoleate → triricinolein.

The major biosynthetic pathway from 2-oleoyl-PC to triacylglycerols containing ricinoleate is shown in Scheme 1.

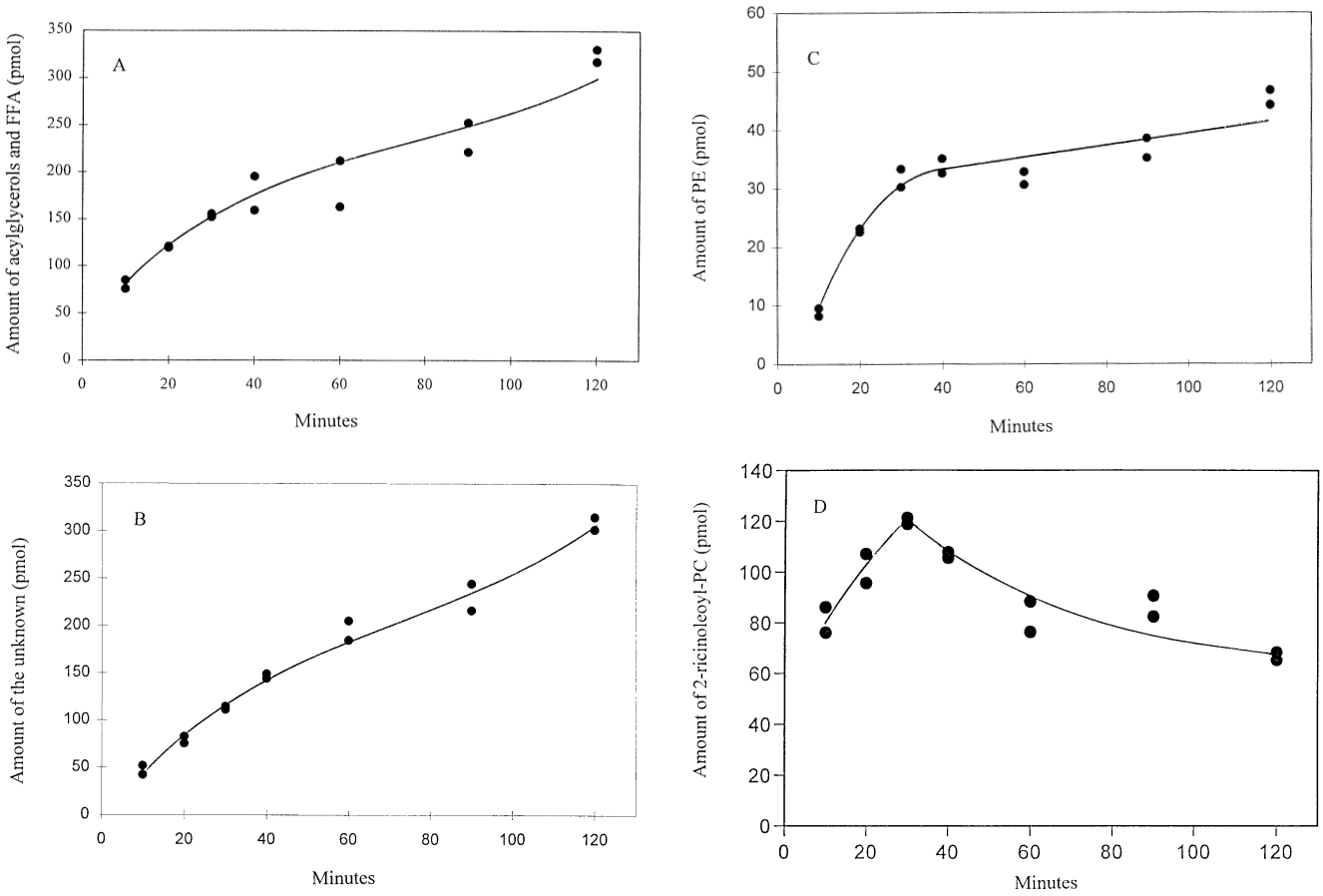


FIG. 6. Amounts of lipid classes shown in Figure 1 formed at various times of castor microsomal incubations with 1-palmitoyl-2-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine. Units for y-axes are the pmol of labeled fatty acids in each lipid class derived from [^{14}C]oleate of the substrate. Figure 6A, acylglycerols and FFA (including triricinolein); Figure 6B, the unknown; Figure 6C, PE; Figure 6D, 2-ricinoleoyl-PC. See Figure 1 for abbreviations.

Step 1: oleoyl-12-hydroxylase, by cytochrome b_5, NADH, O_2, ATP (21). This step is favored between steps 1 and 2. Step 2: oleoyl-12-desaturase, by cytochrome b_5, NADH, O_2, ATP (21). Step 3: phospholipase A_2. Step 4: acyl-CoA synthetase, by ATP, CoA. Step 5: glycerol-3-phosphate acyltransferase, by acyl-CoA. Step 6: lyso-phosphatidic acid acyltransferase. Step 7: phosphatidic acid phosphatase. Step 8: diacylglycerol acyltransferase, by acyl CoA, blocked (completely or partially) when the substrate contains no ricinoleate, in favor of the formation of triricinolein. Because of the block, 1-acyl-2-oleoyl-*sn*-glycerol was accumulated. The conversion of 1,2-diricinoleoyl-*sn*-glycerol to PC by CDP-choline:diacylglycerol cholinephosphotransferase was blocked owing to the presence of membrane-incompatible fatty acid (22). Step 9: phospholipase C. The hydrolysis of PC at the *sn*-2 position by phospholipase A_2 favors ricinoleate as shown in Table 1. Removal of ricinoleate from PC has been explained in part by the action of phospholipid acyl hydrolases in castor microsomes that display strong preference for releasing ricinoleate from the *sn*-1 and *sn*-2 positions of PC (17). The accumulation of 1,2- diricinoleoyl-*sn*-glycerol was not significant as shown in Figure 4C, because it would elute before triricinolein (radioactive peak 1 in Fig. 4C, 7.9 min) and after ricinoleate (peak 5, 2.9 min), and the radioactive peaks at retention times of 3.7 and 4.9 min contained mostly radioactive oleate after the hydrolysis of these two peaks. The conversion from 1-palmitoyl-2-oleoyl-*sn*-glycerol to 1-palmitoyl-2-oleoyl-3-acyl-*sn*-glycerols was blocked (completely or partially) and the enzyme, diacylglycerol acyltransferase, favored the conversion of 1,2-diricinoleoyl-*sn*-glycerol to 1,2-diricinoleoyl-3-acyl-*sn*-glycerols. The diacylglycerol acyltransferase favored the formation of triricinolein and 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol as shown in Figure 4C. The castor endosperm diacylglycerol acyltransferase displays a 3- to 4-fold preference for acylating 1,2-diricinoleoyl-*sn*-glycerol over 1,2-dioleoyl-*sn*-glycerol and exhibits low activity toward diacylglycerols with only one ricinoleate residue (23). In a separate study, the diacylglycerol acyltransferase showed selectivities for substrates containing ricinoleate on both 1,2-diricinoleoyl-*sn*-glycerol and ricinoleoyl-CoA (24). The accumulation of a large quantity of radioactive 1,2-diricinoleoyl-3-oleoyl-*sn*-glycerol in this incubation (Fig. 4C), in contrast to castor oil constituents (Fig. 4A), might be due to the presence of a large quantity of oleoyl-CoA in the incubation which came from the large quantity of 2-oleoyl-PC as the substrate. In this study, we have produced metabolic evidence that three enzymatic steps, oleoyl-12-hydroxylase, phospholipase A_2 and diacylglycerol acyltransferase, drive ricinoleate into triricinolein. The prolonged and

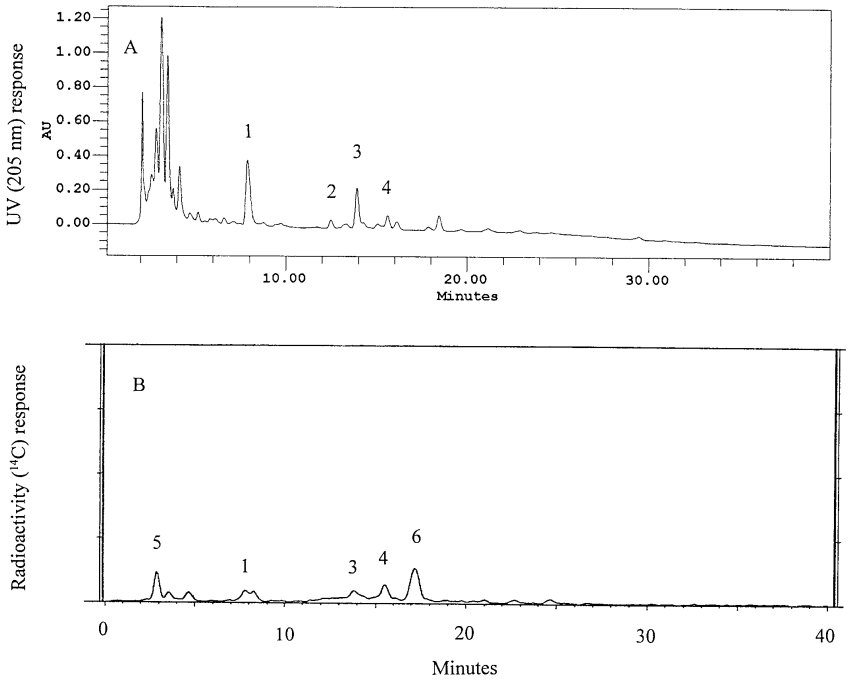


FIG. 7. HPLC identification of acylglycerols in the total lipid extract from the castor microsomal incubation of 1-palmitoyl-2-[^{14}C]ricinoleoyl-*sn*-glycero-3-phosphocholine, using a C_{18} HPLC system (see the Experimental Procedures section, HPLC, v). A linear gradient from 100% methanol to 100% isopropanol in 40 min was used. The sample was cochromatographed with castor oil (80 μg). Peak 1, triricinolein (7.8 min); peak 2, diricinoleoyl-linolenoyl-glycerol (12.4 min); peak 3, diricinoleoyl-linoleoyl-glycerol (13.8 min); peak 4, diricinoleoyl-oleoyl-glycerol (15.6 min); peak 5, ricinoleate (2.9 min); peak 6, 1-palmitoyl-2-oleoyl-*sn*-glycerol (17.2 min). See Figures 1 and 3 for abbreviations.

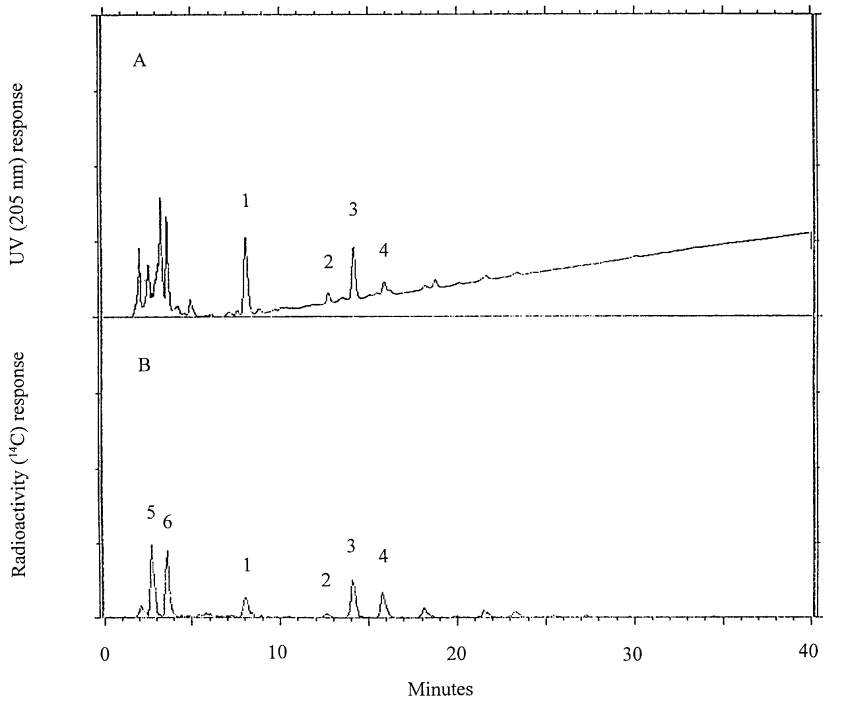


FIG. 8. HPLC identification of acylglycerols in the fraction of acylglycerols and FFA from the castor microsomal incubation of free [^{14}C]ricinoleate using a C_{18} HPLC system (see the Experimental Procedures section, HPLC, v). A linear gradient from 100% methanol to 100% isopropanol in 40 min was used. The sample was cochromatographed with castor oil (80 μg). Peak 1, triricinolein (8.1 min); peak 2, diricinoleoyl-linolenoyl-glycerol (12.6 min); peak 3, diricinoleoyl-linoleoyl-glycerol (14.1 min); peak 4, diricinoleoyl-oleoyl-glycerol (15.8 min); peak 5, ricinoleate (2.7 min); peak 6, may be 1,2-diricinoleoyl-*sn*-glycerol (3.6 min). See Figures 1 and 3 for abbreviations.

increasing activity of oleoyl-12-hydroxylase and diminishing activity of oleoyl-12-desaturase throughout development (5) are also important factors in the production of triacylglycerols containing ricinoleate.

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