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Comparison of the Incorporation of Oleate and Ricinoleate into Phosphatidylcholines and Acylglycerols in Soybean Microsomes

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The incorporations of oleate (endogenous) and ricinoleate (nonendogenous) into phosphatidylcholine (PC) and acylglycerol (AG) in immature soybean microsomes were compared. [^{14}C]Oleate and [^{14}C]-ricinoleate were incubated individually with soybean microsomal preparations for up to 4 h, and molecular species of PC and AG incorporated were identified and quantified by HPLC. The activities of acyl CoA:*lyso*PC acyltransferase and phospholipase A_2 are in general not affected by the fatty acid (FA) chain at the *sn*-1 position. However, comparison between oleate and ricinoleate revealed that different FA incorporated at *sn*-2 of PC showed some different selection of the molecular species of *lyso*PC. The incorporation of [^{14}C]ricinoleate into triacylglycerols (TAG) was slightly better than that of [^{14}C]oleate and indicated that soybean was capable of incorporating ricinoleate into TAG when ricinoleate can be produced endogenously in a transgenic soybean. The incorporation of FA into TAG in soybean microsomes was much slower than that in castor microsomes.

KEYWORDS: Molecular species; phosphatidylcholine; triacylglycerols; ricinoleate; biosynthesis; HPLC; microsomes; soybean

INTRODUCTION

Ricinoleate (R) has many industrial uses such as the manufacture of lithium grease, plastics, coatings, and cosmetics. The only commercial source of ricinoleate is castor oil, which contains 90% of its fatty acids (FA) as ricinoleate (*1*). Because castor bean contains the toxin protein ricin and potent allergens, it is hazardous to grow, harvest, and process. It would be desirable to produce ricinoleate from a transgenic oilseed lacking these noxious components. The cDNA for oleoyl-12-hydroxylase, the enzyme catalyzing the hydroxylation of oleate (O) to ricinoleate, has been cloned from castor and expressed in tobacco (*2*) and *Arabidopsis thaliana* (*3*), but both resulted in low levels of hydroxy FA compared to castor oil (20% hydroxy FA in transgenic *A. thaliana* vs 90% ricinoleate in castor oil). Because production of hydroxy and other polar FA can expand uses of seed oils, we have investigated castor oil biosynthesis, to understand which enzymatic steps contribute to high ricinoleate incorporation in oil (*4*, *5*). Conversely, it is essential to identify enzymatic steps that limit ricinoleate incorporation in oilseeds that do not normally produce ricinoleate. To develop a transgenic plant that produces seed oil containing a high level of ricinoleate, it is important to know the biosynthetic pathways leading to the triacylglycerols (TAG) of castor oil and the seed oil of the plant that is targeted to produce a high level of ricinoleate.

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We have identified and quantified (*6*, *7*) the molecular species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and TAG produced from incubation with labeled ricinoleate, oleate, linoleate (L), linolenate (Ln), stearate (S), and palmitate (P). The most abundant PC molecular species was 1-linoleoyl-2-oleoyl-PC (*6*). We have recently identified and quantified the molecular species of TAG in castor oil, and the major molecular species are RRR (71%), RRO (9%), RRL (7%), and RRS (1%) (*8*).

Although soybean is the major source of vegetable oil in the United States (*USDA Agricultural Statistics*, U.S. Government Printing Office: Washington, DC, 2003), its oil content is only ∼20% compared to 30−50% for other oilseeds (*9*). It is thus of interest to compare its capability for TAG production with that of castor (up to 60% oil content) and to identify enzymatic steps in soy oil biosynthesis that may reflect general steps blocking ricinoleate incorporation. The incorporation of [^{14}C]-oleoyl-CoA into PC in germinated soybean microsomal incubations was previously reported (*10*). The molecular species most actively formed were 1-linoleoyl-2-oleoyl-PC (LO-PC), which represents 50% of the newly synthesized PC, with PO-PC forming 23%. The labeled oleoyl residues were at least 95% esterified at the *sn*-2 position of PC. Because we are interested in the incorporation of FA into TAG, immature soybean microsomes were used in this study instead of the germinated soybean microsomes used earlier (*10*). The molecular species of PC in soybean have been identified and quantified (*11*). The FA components of the PC were linoleate, oleate, palmitate,

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linolenate, and stearate in order of decreasing amount. The FA components of TAG in soybean were in the same order as that of PC (*12*). The major molecular species of TAG in soybean oil (*13*) were identified as LLL (18%, wt %), OLL (15%), PLL (10%), LLLn (8%), and OOL (6%).

MATERIALS AND METHODS

Microsomal Incubation. Microsomes from immature soybean seed (at about the midmaturing period, seed coat removed, mostly cotyledon, *Glycine max* L.) were prepared as previously described (*4*) for castor microsomes (mostly from endosperm). Microsomal incubations (*4*) were scaled up to generate suitable amounts of material. The incubation mixture in a total volume of 5 mL included sodium phosphate buffer (0.1 M, pH 6.3), CoA-SH (2.5 μmol), NADH (2.5 μmol), ATP (2.5 μmol), $MgCl_2$ (2.5 μmol), catalase (5000 units), and microsomal fraction from immature soybean seed (300 μL, 2.18 mg of protein). [^{14}C]Oleate (55 Ci/mol, American Radiolabeled Chemicals, Inc., St. Louis, MO) and [^{14}C]ricinoleate (55 Ci/mol, American Radiolabeled Chemicals, Inc.) were used individually as incubation substrates. The [^{14}C]FA substrates (1.25 μCi, 22.7 nmol, in 100 μL of ethanol) were added last into screw-capped bottles containing incubation mixtures, followed by immediate mixing. The mixtures were then incubated in a shaking water bath for 1, 2, 3, and 4 h at 22 °C. The incubations were stopped by suspension in 18.75 mL of chloroform/methanol (1:2, v/v), followed by mixing with 3.15 mL of chloroform and 3.15 mL of water. The lower chloroform layer containing the lipid extract was dried and fractionated on a silica HPLC system to separate lipid classes as described below.

HPLC. HPLC was carried out on a liquid chromatograph (Waters Associates, Milford, MA), using an absorbance detector (Waters 2487) at 205 nm. Radiolabeled lipids were separated by HPLC and identified by cochromatography with lipid standards by matching the retention times (RT) from the absorbance detector and the flow scintillation analyzer. The flow rate of HPLC eluents was 1 mL/min. The flow rate of liquid scintillation fluid (Ultima Flo M, Packard Instrument Co., Downers Grove, IL) through the flow scintillation analyzer (150TR, Packard Instrument Co.) was 3 mL/min. The flow scintillation analyzer used a 200-μL flow cell.

Separation of Lipid Classes. Lipid classes were separated according to Singleton and Stikeleather (*14*) on a silica column [25 × 0.46 cm, 5 μm, Luna, silica(2), Phenomenex, Torrance, CA] with a linear gradient starting at 2-propanol/hexane (4:3, v/v) to 2-propanol/hexane/water (4:3:0.75, v/v/v) in 20 min and then held isocratically for 20 min. A prepacked silica saturator column (3 × 0.46 cm, 15−25 μm, Phenomenex) was installed between the pump and injector to saturate the mobile phase with silica before it reached the analytical column.

Separation of Molecular Species of PC and PE. Molecular species of PC (*15*) and PE (*16*) were separated as previously reported, using a C_8 column (25 × 0.46 cm, 5 μm, Luna C8, Phenomenex). The separation of molecular species of PC used a 40 min linear gradient of 90% aqueous methanol to 100% methanol, both containing 0.1% of concentrated NH_4OH. The separation of molecular species of PE used a 40 min linear gradient of 88% aqueous methanol to 100% methanol containing 0.1% of concentrated NH_4OH. NH_4OH was used as a silanol-suppressing agent. The PC and PE standards were purchased from Avanti Polar Lipids, Inc. (Albaster, AL) and Sigma (St. Louis, MO).

Separation of Molecular Species of TAG and Diacylglycerols (DAG). Molecular species of TAG and DAG were separated as we previously reported (*17*), using a C_{18} column (25 × 0.46 cm, 5 μm, Ultrasphere C18, Beckman Instruments Inc., Fullerton, CA) with a linear gradient from 100% methanol to 100% 2-propanol in 40 min. The TAG and DAG standards were purchased from Sigma and Nu-Chek Prep, Inc. (Elysian, MN).

Determination of the *sn*-1,2 Positions of [^{14}C]FA on PC. The [^{14}C]-PC obtained from the silica HPLC were hydrolyzed with 0.2 mg of phospholipase A_2 (*Naja mossambica mossambica*, P4034, Sigma) dissolved in a total of 1 mL of buffer [0.1 M, tris(hydroxymethyl)-aminomethane, pH 8.9]. The PC dissolved in 20 μL of ethanol was added to the buffer containing phospholipase A_2, followed by immediate mixing. The mixture was then incubated in a shaking water bath at 25 °C overnight and then neutralized with HCl (0.1 M). The total lipid was extracted as described above for the microsomal incubation, using one-fifth of the quantity of the reagents. The free FA and *lyso*PC formed were separated by silica HPLC, and the radioactivity was measured by flow scintillation analyzer. Under these conditions, the esters at *sn*-2 of PC were completely hydrolyzed by phospholipase A_2. To elute *lyso*PC more rapidly after phospholipase A_2 hydrolysis of PC, a linear gradient of 2-propanol/hexane (4:3, v/v) to 2-propanol/hexane/water (4:3:0.85, v/v/v) in 20 min, then isocratic for 30 min, was used. The eluent B was near saturation with water.

RESULTS AND DISCUSSION

We wanted to compare the capability of castor and soy to incorporate FA normally not produced in their oil. Therefore, [^{14}C]oleate and [^{14}C]ricinoleate were incubated with immature soybean microsomal preparations for 1−4 h. Oleate is one of the main FA in soybean, whereas ricinoleate is not endogenous in soybean. Due to the very low incorporation of label into PC, PE, and AG compared to that of castor microsomal preparations (*6*, *7*), the incubation volume was increased to 5 mL from the 1 mL incubation (*4*, *5*) and the microsome concentration was also increased to ~3.2 times (*4*−*7*). The low incorporation into various lipid classes in soybean microsomes probably reflects the low oil content of these soybeans (15%) to that of castor bean (55−60%) (C. Turner, unpublished data).

The total lipid extracts from the incubations were used for the separation of lipid classes by silica HPLC, and the acylglycerol (AG), PE, and PC fractions in the order of elution were collected (*7*). The collected phospholipase A_2 hydrolysis product of PC showed the incorporation of >95% of the [^{14}C]-oleate and [^{14}C]ricinoleate at the *sn*-2 position of labeled PC, similar to results for the germinated soybean microsomal incubation (*10*) and castor microsomal incubations (*6*).

The molecular species of PC collected from silica HPLC were separated by C_8 HPLC (*15*). They were identified in order of elution as LnO-PC (1-linolenoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine), LO-PC, PO-PC, OO-PC, and SO-PC (**Figure 1A**) from [^{14}C]oleate incubation and RR-PC, LnR-PC, LR-PC, PR-PC, OR-PC, and SR-PC (**Figure 1B**) from [^{14}C]ricinoleate incubation. Identifications were made by cochromatography with available PC standards, OO-PC, PO-PC, and SO-PC, and by relative retention times (RRT) of PC recently reported (*18*). Neither hydroxylation of 2-oleoyl-PC to 2-ricinoleoyl-PC nor desaturation to 2-linoleoyl-PC was observed, in contrast to results with castor (*6*).

Figure 2 shows the levels of the molecular species of PC incorporating [^{14}C]oleate and [^{14}C]ricinoleate at various incubation times. The ratios of the same molecular species at four different times were similar, indicating that the activities of acyl-CoA:*lyso*PC acyltransferase (EC 2.3.1.23) and phospholipase A_2 (EC 3.1.1.4) were not affected in general by the difference in acyl chain at the *sn*-1 position of *lyso*PC and PC, respectively, in accordance with results observed as for germinated soybean microsomes (*10*) and castor microsomes (*6*). Demandre et al. (*10*) have shown the nonselective acylation in general for *lyso*PC molecules in germinated soybean microsomal incubation with oleoyl-CoA. The levels of LO-PC and OO-PC (**Figure 2A**) were higher than those of LR-PC and OR-PC (**Figure 2B**). The level of LnO-PC (**Figure 2A**) was higher than that of LnR-PC (**Figure 2B**). Thus, comparison between oleate and ricinoleate revealed that the different FA incorporated at *sn*-2 showed some selection of the molecular species of *lyso*PC.

The levels of the molecular species of PC incorporating [^{14}C]-oleate were higher than those incorporating [^{14}C]ricinoleate

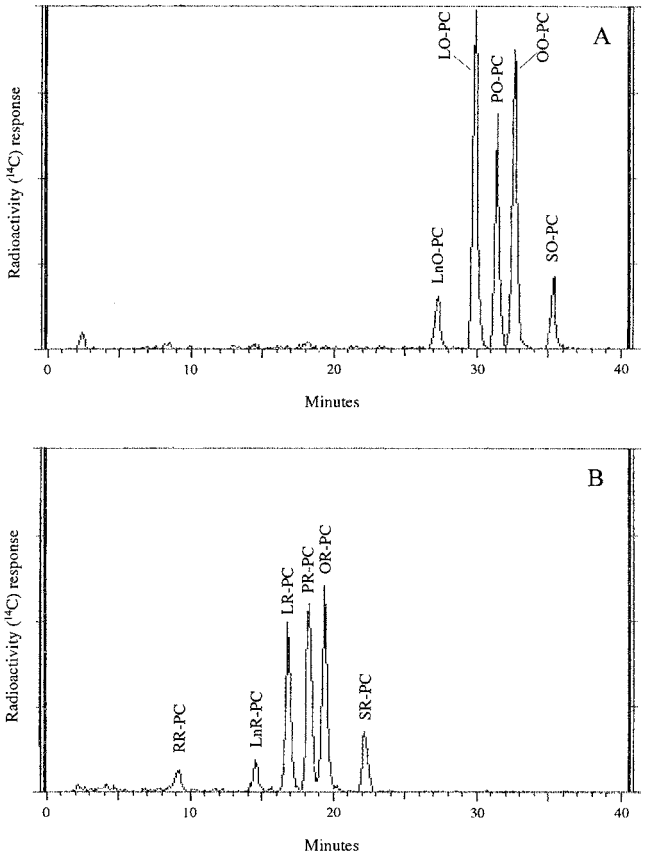

Figure 1. C_8 HPLC radiochromatograms for the separation of the molecular species of PC incorporating [^{14}C]oleate (**A**) and [^{14}C]ricinoleate (**B**) in immature soybean microsomal incubations (2 h). Half of the total PC was used for this C_8 HPLC. For the HPLC conditions see Material and Methods. The molecular species of PC are designated on the peaks with the two acyl chains as R (ricinoleate), S (stearate), O (oleate), L (linoleate), Ln (linolenate), and P (palmitate); the first letters are at *sn*-1 positions, and the second letters are at *sn*-2 positions.

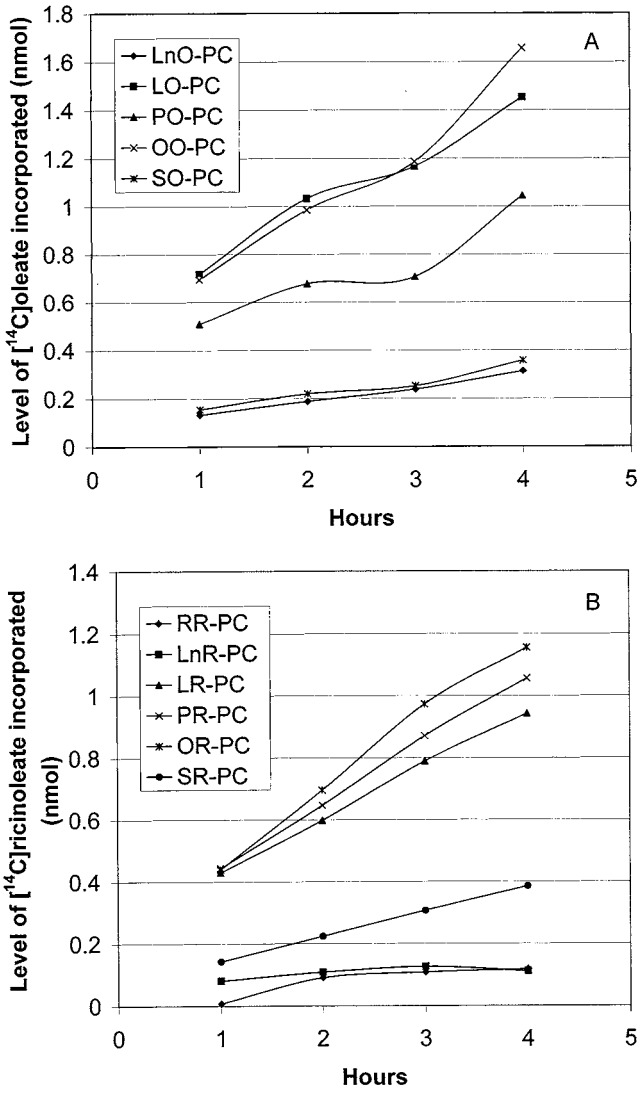

Figure 2. Time courses of the incorporations of [^{14}C]oleate (**A**) and [^{14}C]-ricinoleate (**B**) into the molecular species of PC in immature soybean microsomes. For the designation of the molecular species of PC, see the caption of **Figure 1**.

(**Figure 2**). The accumulation of 2-oleoyl-PC and 2-ricinoleoyl-PC can also be affected by the activity of phospholipase A_2, which is generally more active on 2-ricinoleoyl-PC (turnover) in microsomes of higher plants than on 2-oleoyl-PC (*4*, *19*, *20*). In our previous study of the incorporation of various FA into PC in castor microsomal incubations, the levels of various molecular species of PC reached equilibrium in 1 h when the rate of incorporation from FA became equal to the rate of hydrolysis by phospholipase A_2 (*6*). In the soybean microsomes, levels of 2-oleoyl-PC and 2-ricinoleoyl-PC increased continuously in 4 h of incubations in general (**Figure 2**), suggesting a very slow incorporation and turnover.

The incorporation of FA into PC in immature soybean microsomes (**Figure 2**) was lower than that of castor microsomes (*6*). The total labeled PC produced in a 2 h soybean microsomal incubation (using 2.18 mg of microsomes protein in a 5 mL incubation) of [^{14}C]oleate (**Figure 2A**) was 3.10 nmol; the total labeled PC from a 1 h castor microsomal incubation (2.76 mg of microsomes protein in a 20 mL incubation) with [^{14}C]oleate was 64.0 nmol (*6*). The incorporation of [^{14}C]oleate into PC in castor microsomes was 32.6 times that in soybean microsomal incubation. The total PC in the soybean microsomal incubation of [^{14}C]ricinoleate (**Figure 2B**) was 2.4 nmol, whereas that in castor microsomal incubation was 19.4 nmol (*6*). The incorporation activity from [^{14}C]ricinoleate into PC in castor microsomes was 12.8 times that in soybean microsomes. Molecular species of PE incorporating [^{14}C]oleate and [^{14}C]-ricinoleate in immature soybean microsomes were not detected despite the presence of PE in soybean (*11*).

Figure 3 shows the separations of the molecular species of AG incorporating [^{14}C]oleate and [^{14}C]ricinoleate. The incorporation of FA and acyl CoA into the molecular species of TAG and DAG in soybean microsomes has not been previously reported. TAG incorporating [^{14}C]oleate were LLLn, LLL, OLL, OOL, OOO, and OOS in the order of elution (**Figure 3A**). Among these, TAG, LLL, OLL, OOL, and OOO were identified tentatively by cochromatography with standards. LLLn was designated by the contribution of functional groups of FA to the RRT of TAG (*21*). All of these TAG in soybean oil have previously been identified by HPLC coupled with atmospheric pressure chemical ionization mass spectroscopy (*12*). The DAG, OO, was identified by cochromatography with the standard, and OL was designated by its RRT published recently (*18*). In the HPLC system of **Figure 3**, the RRT were 16.3 min (1,2-dioleoylglycerol) and 16.2 min (1,3-dioleoylglycerol) (*17*). These regioisomers could not be separated well by this HPLC system but can be separated by a CN column (*22*). Monooleoylglycerol was not detected by the HPLC system for free

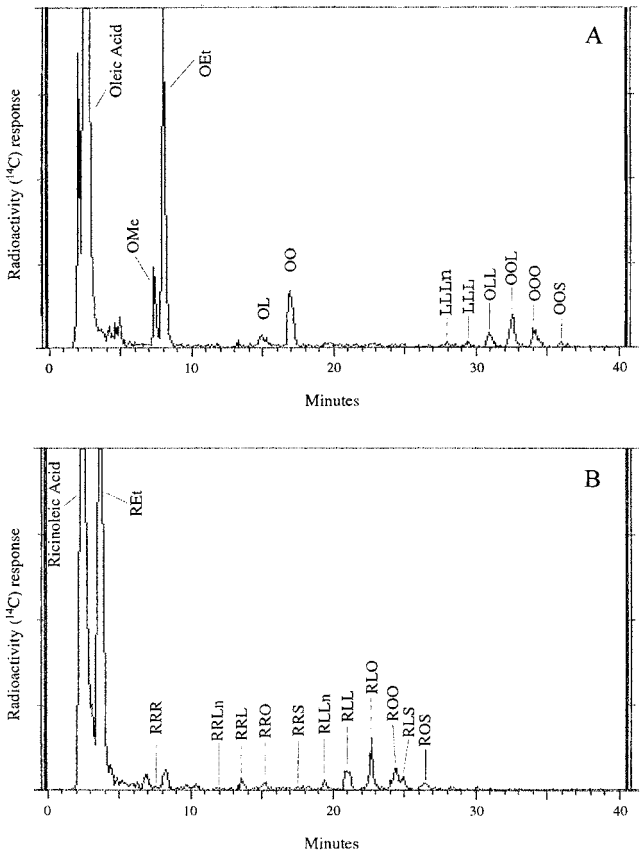

Figure 3. C_{18} HPLC radiochromatograms for the separation of the molecular species of AG incorporating [^{14}C]oleate (**A**) and [^{14}C]ricinoleate (**B**) in immature soybean microsomal incubations (2 h). Half of the total AG was used for this C_{18} HPLC. For the HPLC conditions see Material and methods. The molecular species of AG are designated on the peaks as R (ricinoleate), S (stearate), O (oleate), L (linoleate), Ln (linolenate), and P (palmitate). OMe is oleoic acid methyl ester, OEt is oleic acid ethyl ester, RMe is ricinoleic acid methyl ester, and REt is ricinoleic acid ethyl ester.

FA (*23*). The RRT in this HPLC system were 22.1 min (1-monooleoyl-glycerol), 20.7 min (2-monooleoylglycerol), and 27.1 min (oleate).

Molecular species of TAG containing oleate and TAG containing palmitate cannot be separated as well in this C_{18} HPLC system as the RRT previously reported (*17*): 32.8 min (OOO), 32.9 min (OOP), 33.1 min (OPP), and 33.3 min (PPP). Because OOO and OOP in soybean oil have been identified and quantified by mass spectrometry (*12*, *13*) and palmitate constitutes ~10% of the FA in soybean TAG (*13*), peak OOO shown in **Figure 3A** also contains labeled OOP as a minor component of the peak. Labeled OPP may also be present in the peak of OOO. For the same reasons (*12*, *13*, *17*), peak OOL in **Figure 3A** also contains labeled POL as a minor component in the peak OOL.

Oleic acid ethyl ester (OEt), oleic acid methyl ester (OMe), and oleic acid in **Figure 3A** were identified by cochromatography with standards (Nu-Chek Prep, Inc.) using the C_{18} HPLC systems for TAG (*17*) and for free FA (*23*). The RRT in the C_{18} HPLC for free FA (*23*) were 38.2 min (OEt), 35.9 min (OMe), and 27.1 min (oleic acid). OEt and OMe in **Figure 3A** were not the metabolites of [^{14}C]oleate. The [^{14}C]oleate used for incubation was stored in ethanol by the vendor, and 13% of the radioactivity was associated with OEt determined by the C_{18} HPLC for free FA (*23*) using a flow scintillation analyzer. Some of the [^{14}C]OEt in the incubation substrate was hydrolyzed to [^{14}C]oleate. No [^{14}C]OMe was detected in this commercial [^{14}C]oleate; however, methanol was used in the extraction and in the storage of the extract and AG fractions. The presence of [^{14}C]OMe in **Figure 3A** was likely due to the contact with methanol after the incubation.

Figure 3B shows the molecular species of TAG incorporating [^{14}C]ricinoleate in immature soybean microsomes identified by cochromatography with castor oil. Molecular species of castor oil on the C_{18} HPLC chromatogram have been identified (*8*). The TAG incorporated from [^{14}C]ricinoleate (**Figure 3B**) were RRR (RO), RRLn, RRL, RRO, RRS, RLLn, RLL, RLO, ROO, RLS, and ROS. The RRT of RRR and RO (a DAG) were the same (*18*), and the small peak at 7.5 min may be RRR and/or RO. RL was not identified and should be at 6.1 min. The peaks at 6.9 and 8.3 min were unknown to us. Ricinoleic acid, ricinoleoyl methyl ester (RMe, minor), and ricinoleic acid ethyl ester (REt) were identified by cochromatography with standards using the C_{18} HPLC system for free FA (*23*). The RRT of ricinoleic acid, RMe, and REt in this C_{18} HPLC system were 8.9, 13.6, and 15.9 min, respectively. REt in **Figure 3B** was not a metabolite of [^{14}C]ricinoleate but came from the substrate used. The substrate, [^{14}C]ricinoleate, was also dissolved in ethanol by the vendor and contained 16% of [^{14}C]REt. The [^{14}C]-RMe identified in the C_{18} HPLC for free FA (*23*) was likely from contact with methanol after the incubation. 1,2-Diricinoleoylglycerol (RR) was coeluted with REt in **Figure 3A**. A very low level of 1,2-RR was identified by cochromatography with a standard using the C_{18} HPLC system for free FA (*23*). The RRT in this HPLC system (*23*) were 29.4 min (1,2-RR), 29.9 min (1,3-RR), and 8.9 min (ricinoleate).

The levels of [^{14}C]oleate incorporation into AG after 2 h of incubation were, in the order of elution (**Figure 3A**), 25 pmol (OL), 121 pmol (OO), 9.3 pmol (LLLn), 13 pmol (LLL), 32 pmol (OLL), 66 pmol (OOL), 44 pmol (OOO), and 8.7 pmol (OOS). The major TAG labeled with ^{14}C were OOL, OOO, and OLL, and the major DAG was OO. Total incorporation into TAG was 173 pmol, and that into DAG was 147 pmol. The incorporation level in our previous castor microsomal incubation of [^{14}C]oleate (*7*) was ~15 nmol of TAG and ~4 nmol of DAG. The labeling of TAG in castor microsomal incubation was ~137 times that of soybean microsomal incubation, whereas that of DAG was ~43 times. DAG and TAG levels from the incorporation of [^{14}C]oleate in soybean microsomal incubation were similar (**Figure 3A**), whereas the DAG labeling was one-fourth of TAG in castor microsomal incubation. This suggests that the acylation of DAG to TAG is blocked in soybean microsomes, leading to accumulation of the intermediate on the pathway, DAG. In the time course study (up to 4 h), the levels of molecular species of TAG and DAG increased continuously, probably due to the slow incorporation. In our recent castor microsomal incubation (up to 120 min) of [^{14}C]-oleate (*7*), the labeling of the molecular species of TAG leveled off at 60 min in general, whereas the level of OO continued increasing beyond 120 min.

The levels of [^{14}C]ricinoleate incorporation into AG in soybean microsomal incubation were, in the order of elution, 5 pmol (RR), 5 pmol (RRR and/or RO), 18 pmol (RRL), 20 pmol (RRO), 21 pmol (RLLn), 46 pmol (RLL), 84 pmol (RLO), 52 pmol (ROO), 17 pmol (RLS), and 20 pmol (ROS), and the total of identified TAG was 283 pmol. The major TAG species were RLO, ROO, and RLL. The total TAG level incorporating [^{14}C]-ricinoleate was slightly higher than that from [^{14}C]oleate (173 pmol) in the soybean microsomal incubation. However, the level

of DAG, RR (5 pmol), incorporating [^{14}C]ricinoleate in soybean microsomal incubation was much lower than the level of OO (121 pmol) incorporating [^{14}C]oleate. The TAG incorporation level of [^{14}C]ricinoleate in castor microsomal incubation (*7*) was ~270 times that in soybean microsomal incubation. The levels of the molecular species of AG continued to increase throughout the 4 h soybean microsomal incubation, whereas the levels of TAG labeled in castor microsomal incubation (*7*) flattened at 60 min in general, while the level of RR increased continuously for 120 min. This again may be due to the slow incorporation of FA into TAG in immature soybean microsomes.

Conclusion. Production of high levels of ricinoleate in transgenic plants has not been successful, probably due to disruption of membrane function by high ricinoleate. Using soy microsomes as a model system, we have demonstrated the accumulation of 2-ricinoleoyl-PC, which would be a membrane component. Although oilseeds have been demonstrated to have phospholipase A_2 that can remove the ricinoleate, apparently the phospholipase A_2 for 2-ricinoleoyl-PC is far more effective than that of 2-oleoyl-PC. Interestingly, at low levels, using endogenous DAG substrate, soy microsomes do not seem to discriminate between oleate and ricinoleate in the final acylation steps.

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