Triacylglycerol Bioassembly in Microspore-Derived Embryos of Brassica napus L. cv Reston

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ABSTRACT

Eruvic acid (22:1) was chosen as a marker to study triacylglycerol (TAG) biosynthesis in a Brassica napus L. cv Reston microspore-derived (MD) embryo culture system. TAGs accumulating during embryo development exhibited changes in acyl composition similar to those observed in developing zygotic embryos of the same cv, particularly with respect to erucic and eicosenoic acids. However, MD embryos showed a much higher rate of incorporation of 13C-erucyl moieties into TAGs in vitro than zygotic embryos. Homogenates of early-late cotyledonary stage MD embryos (14–29 days in culture) were assessed for the ability to incorporate 22:1 and 18:1 (oleoyl) moieties into glycerolipids. In the presence of [1-14C]22:1-coenzyme A (CoA) and various acyl acceptors, including glycerol-3-phosphate (G-3-P), radiolabeled erucyl moieties were rapidly incorporated into the TAG fraction, but virtually excluded from other Kennedy Pathway intermediates as well as complex polar lipids. This pattern of erucyl incorporation was unchanged during time course experiments or upon incubation of homogenates with chemicals known to inhibit Kennedy Pathway enzymes. In marked contrast, parallel experiments conducted using [1-14C]18:1-CoA and G-3-P indicated that 14C oleoyl moieties were incorporated into lysophosphatidic acids, phosphatidic acids, diacylglycerols, and TAGs of the Kennedy Pathway, as well as other complex polar lipids, such as phosphatidylethanolines and phosphatidylethanolamines. When supplied with [2-3H(N)]G-3-P and [1-14C]22:1-CoA, the radiolabeled TAG pool contained both isotopes, indicating G-3-P to be a true acceptor of erucyl moieties. Radio-high-performance liquid chromatography, argentation thin-layer chromatography/gas chromatography/mass spectrometry, and stereospecific analyses of radiolabeled TAGs indicated that 22:1 was selectively incorporated into the sn-3 position by a highly active diacylglycerol acyltransferase (DGAT; EC 2.3.1.20), while oleoyl moieties were inserted into the sn-1 and sn-2 positions. In the presence of sn-1,2-dienoyl and [1-14C]22:1-CoA, homogenates and microsomal preparations were able to produce radiolabeled trirucin, a TAG not found endogenously in this species. A 105,000g pellet fraction contained 22:1-CoA:DGAT exhibiting the highest specific activity. The rate of 22:1-CoA:DGAT activity in vitro could more than account for the maximal rate of TAG biosynthesis observed in vivo during embryo development. In double label experiments, G-3-P was shown to stimulate the conversion of [3H]phosphatidylcholines to [3H]diacylglycerols, which subsequently acted as acceptors for 13C erucyl moieties. In vitro, 22:1 moieties did not enter the sn-1 position of TAGs by a postsynthetic modification or transacylation of preformed TAGs.

In higher plants, 22:1 is seed-specific and is confined almost exclusively to the neutral lipid fraction in developing oilseeds. It is not known to be a significant component of membrane lipids (22, 38). Thus, 22:1 is an ideal marker for investigating metabolic pathways involved in storage lipid biosynthesis. Furthermore, seed oils high in 22:1 are desirable as industrial feedstocks for the production of high temperature lubricants, nylon, and plasticizers (37), and the existence of a much larger overall market potential for products based on such oils has been cited (51). Although it is generally accepted that in higher plants, C16 and C18 fatty acyl moieties are incorporated into TAGs via the G-3-P pathway according to Kennedy (6, 44), at present, the mechanism involved in the biosynthesis of TAGs containing 22:1 is not fully understood, despite several studies aimed at elucidating the pathway in a number of developing oilseeds (7, 8, 20, 28, 34, 35, 45).

* Abbreviations: 22:1, erucic acid. (E), cis-Δ-13-docosenoic acid; TAG, triacylglycerol; 2-Bro, 2-bromooctanoic acid; CPTase, CDP-choline (1,2-diacetylglycerol cholinephosphotransferase [EC 2.7.8.2]); DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase (EC 2.3.1.20); DHAP, dihydroxyacetone phosphate; DIPFP, di-isopropyl fluorophosphate; E1, electron impact; FAME, fatty acid methyl ester; FFA, free fatty acid; G-3-P, glycerol-3-phosphate; G-3-P AT, glycerol-3-phosphate acyltransferase (EC 2.3.1.15); LPA, lysophosphatic acid; LPAT, lysophosphatic acid acyltransferase (EC 2.3.1.51); LPC, lysophosphatidylcholine; MAG, monoaecylglycerol; MD, microspore-derived; PA, phosphatic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; 18:1, oleic acid (O), cis-Δ-9-octadecenoic acid; 20:1, (E), cis-Δ-11-eicosenoic acid; 18:2, linoleic acid, (L), cis-Δ-9,12-octadecadienoic acid; 18:3, α-linolenic acid, (Ln), cis-Δ-9,12,Δ-15-octadecatrienoic acid; 16:0, palmitic acid, (P), hexadecanoic acid (all other fatty acyl groups are designated by number of carbons: number of double bonds): EOE, erucyl oleoyl erucylglycerol; LnOE, 18:3/18:1/22:1; LOE, 18:2/18:1/22:1; OOE, 18:1/18:1/22:1; POE, 16:0/18:1/22:1; LOL, 18:2/18:1/18:2; LOO, 18:2/18:1/18:1; OOO, triolein.

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Few dicotyledonous oilseed crops have been amenable to the induction of high frequencies of embryogenesis in vitro. However, early studies using small numbers of anther-derived embryos of *Brassica campestris* revealed that 22:1 was present, and it was suggested that such gametophytic embryos might be utilized to study storage lipid biosynthesis (18). MD embryos have been obtained from several plant species, including *Brassica napus* (19), *Hordeum vulgare* (21), and *Nicotiana rustica* (46). Although the process is genotype-dependent, it is possible to produce thousands of embryos at particular developmental stages from microsomes isolated from immature inflorescences of certain *B. napus* genotypes (19, 33, 36).

In our laboratories, we are interested in the analysis of seed-specific processes associated with the accumulation of storage lipids, particularly those rich in 22:1, in *Brassica*, and other oilseed species. A greater understanding of these processes may permit modification of seed oil biosynthesis, both quantitatively and qualitatively, through the expression of foreign genes in a seed-specific manner. Thus, we have been investigating the *B. napus* MD embryo system as a possible model for oilseed development. Preliminary investigations into lipid biosynthesis in the MD system have shown these haploid embryos to be very active in the synthesis and metabolism of erucyl moieties (48, 50). Here, we present evidence that MD embryos from a high 22:1 variety (cv Reston) of *B. napus* contain storage lipids that have a fatty acyl composition similar to zygotic embryos and yield highly active cell-free enzyme preparations and microsomal fractions for *in vitro* studies of the bioassembly of TAGs, particularly those containing 22:1. A highly active erucyl-CoA:DGAT is identified in the MD embryo system.

**MATERIALS AND METHODS**

**Lipid Substrates and Biochemicals**

[1-14C]22:1 (52 mCi·mmol⁻¹) and [1-14C]18:1 (58 mCi·mmol⁻¹) were purchased from New England Nuclear Research Products (Mississauga, ON) and Amersham Canada Ltd. (Oakville, ON), respectively, and converted to [1-14C]erucyl-CoA and [1-14C]oleoyl-CoA by an enzymatic method described previously (49). [2-14C]Malonyl-CoA (55 mCi·mmol⁻¹) and [1-14C]trioleoyl-glycerol (112 mCi·mmol⁻¹) were purchased from Amersham, while 1-[2-3H(N)]G-3-P (10.6 Ci·mmol⁻¹) was acquired from New England Nuclear.

Dierucin and diolein, each enriched in the sn-1,2 isomer (NuChek Prep Inc., Elysian, MN), were purified and separated from their sn-1,3 isomers by TLC on Silica 60 G (E. Merck, Darmstadt, FRG) impregnated with 10% w/w boric acid (16). The 1,2-DAGs were scraped from the plates and eluted from the silica with water-saturated diethyl ether. The ether extract was backwashed with ice water to remove traces of boric acid and then dried over anhydrous sodium sulfate. After removing the ether under a stream of nitrogen, the DAGs were redissolved in hexane and stored at -80°C.

1,2-Dioleoyl,3-erucaglycerol was synthesized from 1,2-diolein (15 μmol) by condensation with erucyl chloride (20 μmol) in the presence of 0.75 mL benzene:pyridine (1:1, v/v). After stirring the reaction mixture under nitrogen for 18 h at 50°C, most of the solvent was evaporated, 0.2 mL water was added, and the TAG extracted with hexane. The hexane extract was backwashed repeatedly with 0.5 mL NaHCO₃ to remove traces of pyridine. The TAG product was purified by TLC on Silica Gel G in hexane:diethyl ether (80:20, v/v) and the structure of the intact TAG (Mᵋ = 941) confirmed by direct probe EI-MS (see below). In addition, the acyl composition was checked by transmethylation followed by GC analysis of FAMEs (see below). Numerous other racemic mixed-acyl TAGs, not commercially available, were required as standards for HPLC and MS. These were synthesized, purified, and characterized by similar procedures using the necessary mono- or diacylglycerols and acyl chlorides as starting materials.

1,2-Dierucoyl-PA was synthesized via the corresponding PC as follows: 1,2-dierucin (10 μmol) was reacted with 0.65 mL CH₂Cl₂:pyridine:phosphorous oxychloride (95:95:10, v/v/v), with stirring, for 1 h at 4°C, followed by 1 h at 25°C. Dry choline chloride (200 mg) was added, and the reaction mixture was stirred overnight at 30°C. Water (20 μL) was added, and the reaction stirred an additional 30 min at 30°C. Solvents were evaporated, and the 1,2-dierucoyl-PC was extracted into 12 mL CH₂Cl₂:MeOH:water:acetic acid (50:39:10:1, v/v/v/v) and partitioned with 4 mL 4 N amonia. After centrifugation, the lower organic layer was saved, the aqueous phase re-extracted with CH₂Cl₂:MeOH:water:acetic acid (50:39:10:1, v/v/v/v) and partitioned with 4 mL 4 N amonia. After centrifugation, the lower organic layer was saved, the aqueous phase re-extracted with CH₂Cl₂:MeOH:water:acetic acid (50:39:10:1, v/v/v/v) and partitioned with 4 mL 4 N amonia. After centrifugation, the lower organic layer was saved, the aqueous phase re-extracted with CH₂Cl₂:MeOH:water:acetic acid (50:39:10:1, v/v/v/v) and partitioned with 4 mL 4 N amonia. After centrifugation, the lower organic layer was saved, the aqueous phase re-extracted with CH₂Cl₂:MeOH:water:acetic acid (50:39:10:1, v/v/v/v) and partitioned with 4 mL 4 N amonia. After centrifugation, the lower organic layer was saved.

All other neutral lipid substrates were obtained from NuChek Prep Inc. 2-BrO was purchased from Dixon Chemicals (Sherwood Park, Alberta, Canada). All polar lipids, non-radioactive acyl-CoAs, di-isopropylfluorophosphate, and most other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC-grade solvents (Omni-Solv, BDH Chemicals) were used throughout these studies.

**Plant Material and Microspore Culture**

*Brassica napus* L. cv Reston, a high erucic acid variety accumulating both C₃₀ and C₃₂ fatty acids in developing seeds, was acquired from the University of Manitoba (Winnipeg, Manitoba, Canada). Reston plants were grown in growth chambers, under a regime of 20°C d (16 h at 400 μE·m⁻²·s⁻¹)/15°C nights (8 h darkness) for 6 weeks and then transferred to 10°C d/5°C nights under the same light conditions for an additional 4 to 6 weeks.

Microspores were isolated and cultured as described previously (50). MD embryos at particular morphogenetic stages (late cotyledonary, early cotyledonary, torpedo, heart, globular) were readily obtained by sequential filtration of cultures through sterilized nylon mesh with pore sizes of 500, 250, 125, 62, and 44 μm, respectively.

For comparison, developing zygotic embryos were selected from selfed Reston flowers at certain times postanthesis, such
that they were morphogenetically similar to the MD embryos. Siliques were surface-sterilized with commercial bleach (20% stock concentration) for 30 min and rinsed with sterile deionized water. Seeds were removed from the siliques, and zygotic embryos were dissected from the seeds under aseptic conditions.

**Analysis of Endogenous Embryo Lipids for Acyl Composition**

At various stages during microspore culture (culture initiation, globular [7–8 d], heart [10 d], torpedo [12 d], early- [14 d], mid- [21 d], late- [28–30 d], and very late- [35 d] cotyledonary stages) or zygotic embryo development (heart [9 d postanthesis], torpedo [12 d postanthesis], early- [16 d postanthesis], mid- [20 d postanthesis], late- [26 d postanthesis], and very late- [32 d postanthesis] cotyledonary stages to the mature seed stage), the acyl composition of embryo lipids was examined. Total acyl lipids were extracted by the method of Hara and Radin (24), a portion of the lipid extract was transmethylated in the presence of 1.5 M methanolic HCl (12), and the acyl composition determined by analysis of the FAMEs on a Hewlett Packard model 5890 gas chromatograph fitted with a DB-23 column (30 m × 0.25 mm, film thickness 0.25 μm; J & W Scientific, Folsom, CA). GC conditions were as follows: injector temperature, 250°C; flame ionization detector temperature, 250°C; running temperature program, 180°C for 1 min, then increasing at 4°C/min to 240°C and holding at this temperature for 10 min.

MD embryos at a late cotyledonary stage (29 d in culture) were analyzed further with respect to the acyl composition of various lipid species. Using a total lipid extract prepared as described above, polar and neutral lipid species were separated by TLC on 0.3-mm Silica Gel 60 H plates developed 4 cm in diethyl ether (to resolve MAGs from polar lipids), air dried, and then developed fully in hexane:diethyl ether:acetic acid (70:30:1, v/v/v). The polar lipids at the origin were scraped and eluted from the silica with a modified (acidic) Bligh and Dyer (9) solution, CH₂Cl₂:MeOH:1 M KCl in 0.2 M H₂PO₄ (1:2:0.8, v/v/v). Phases were separated by adding 2 mL CH₂Cl₂; followed by 2 mL 1 M KCl in 0.2 M H₂PO₄, and the organic phase was removed and dried down under a stream of nitrogen, yielding the polar lipid fraction. TLC regions containing resolved MAGs, DAGs, TAGs, and FFAs were scraped, the lipid species was eluted with water-saturated diethyl ether, and the solvent was then removed under nitrogen. The acyl composition of these polar and neutral lipid species was determined by transmethylation followed by analysis of the resulting FAMEs by GC as described above. Intact TAGs were also analyzed by treatment with pancreatic lipase (16).

**Direct Probe-MS, MS/MS, and GC-MS Analyses of Intact Neutral Lipids**

Both endogenous TAGs, isolated from MD embryos as described above, and 14C-labeled TAGs, products of in vitro biosynthesis experiments (described below), were analyzed by direct probe-MS using a VG 70–250 SEQ hybrid mass spectrometer (VG Analytical, Wythenshawe, Manchester, UK) in the EI mode at an electron energy of 70 eV. Samples were introduced through the solids probe, which was then heated to 330°C. The source temperature was 250°C. Spectra were acquired by scanning from m/z 1300 to m/z 100 every 10 s, with a 0.5-s settling time. Daughter ion spectra were generated in the third field-free region with argon as the collision gas. The pressure in the collision cell was 5 × 10⁻³ torr, and the collision energy was 15 to 20 V.

TAGs were also analyzed by GC-MS on a Hewlett Packard model 5890 gas chromatograph interfaced to the VG 70-SEQ hybrid mass spectrometer. The GC was fitted with a Chrompack TAG Analysis Phase column (25 μ m × 0.25 mm, film thickness 0.1 μ m; 50% phenyl:50% methyl polysiloxane; Chrompack, Middelburg, The Netherlands). GC conditions were as follows: cold on-column injection; oven temperature 360°C, isothermal; carrier gas, helium 50 cm/s. Mass spectrometer conditions were as follows: EI mode, at an electron energy of 70 eV; source temperature 235°C; re-entrant temperature 400°C. Spectra were acquired by scanning from m/z 1300 to m/z 100 every 3 s/decade, with a 0.5-s settling time.

DAGs were silylated using 20 μL N,O-bis(trimethylsilyl)acetamide (Pierce Chemical Co., Rockford, IL) in the presence of 100 μL dry CH₃Cl, and analyzed by GC/MS in the EI mode, on a Finnigan/MAT 4500 instrument (Finnigan Mat, San Jose, CA) fitted with the Chrompack TAG Analysis Phase column. GC conditions were as follows: samples injected in the splitless mode, with the splitter closed for 0.8 min; injector temperature 360°C; oven temperature program, from 200° to 350°C at 4°C/min and held at 350°C for 30 min; carrier gas helium, 88 cm/s measured at 300°C. Mass spectrometer conditions were as follows: EI mode, at an electron energy of 70 eV; source temperature 235°C; transfer oven temperature 350°C; scanned repetitively from m/z 43 to m/z 900 every 2.5 s.

**Preparation of Homogenate and Differential Centrifugation Fractions**

Mid-late cotyledonary stage MD embryos were harvested, rinsed thoroughly with distilled water, and gently suction-filtered to remove excess water. After weighing, embryos (1–3 g fresh weight) were homogenized at 4°C by gentle grinding with a mortar and pestle in the presence a small amount of acid-washed silica sand and 100 mm Hepes-KOH, pH 7.4, containing 0.32 mM sucrose, 1 mM EDTA, and 1 mM DTT. A ratio of 4 to 5 mL grinding medium·g⁻¹ fresh weight was used. The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA) and the cell-free extract was utilized directly for in vitro studies of lipid biosynthesis. Subcellular fractionation of the homogenate was performed by differential centrifugation. All g forces cited are average values. The homogenate was centrifuged at 10,000 × g for 20 min using a Sorvall model RC2-B centrifuge equipped with a Sorvall SM-24 rotor (Ivan Sorvall, Inc., Norwalk, CT). The floating oil bodies were scooped up with the aid of a sterilized inoculum loop, and the residual fat pad material was recovered by aspiration. The oil bodies were resuspended in 5 to 6 mL grinding medium, overlayed with an equal volume of grinding medium containing 0.1 mM sucrose, and recently centrifuged at 18,000g for 15 min. This process of washing the oil
bodies by floating them through a 0.1 m sucrose pad was repeated at least three times, or until the oil body fraction was creamy white in color. After the final centrifugation, the washed oil bodies were scooped up, resuspended in 1 to 2 mL grinding medium, and gently dispersed with a ground glass homogenizer. The original 10,000g pellet was washed by resuspension in 10 mL grinding medium followed by recentrifugation at 10,000g for 20 min. After two such washes, the final pellet was resuspended in 2 mL grinding medium. The 10,000g supernatant was centrifuged on a Beckman model L2-65B ultracentrifuge using a SW 40Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 105,000g for 90 min. The pellet (microsomal fraction) was resuspended in 1 to 2 mL of grinding medium, while the supernatant (soluble protein fraction) was assayed directly. Proteins were estimated by the method of Bradford (10).

Lipid Biosynthesis Studies

Initial studies comparing the incorporation of erucyl and oleoyl moieties into acyl lipids were conducted in standard reaction mixtures containing 80 mM Hepes-KOH (pH 7.4), 1 mM ATP, 0.3 mM CoASH, 2 mM MgCl₂, 200 μM G-3-P, 0.2 to 0.4 mg homogenate protein, and either 40 nCi [1-¹⁴C]erucyl-CoA (10 nCi⋅nmol⁻¹) or 40 μM [1-¹⁴C]oleoyl-CoA (10 nCi⋅nmol⁻¹) in a final volume of 0.5 mL. All assays were carried out at 30°C with shaking (100 rpm) for 5 to 60 min. Protein fractions obtained by differential centrifugation were assayed under the same conditions, utilizing 100 to 200 μg of protein. Reactions were stopped by adding 2 mL CH₂Cl₂:methanol (1:2, v/v), and the resulting mixture was allowed to sit at room temperature for 30 min with occasional vortexing. The organic and aqueous phases were separated by adding 2 mL CH₂Cl₂ followed by 2 mL 1 M KCl in 0.2 M H₃PO₄. After centrifuging, the lower organic phase (containing the total acyl lipid fraction) was saved, the aqueous phase (containing unreacted acyl-CoAs) was backwashed twice with 2 mL CH₂Cl₂, the organic phases were combined, and the solvent was removed under a stream of nitrogen at 35°C.

The radiolabeled products were isolated by TLC. The total acyl lipid extract was dissolved in about 200 μL CH₂Cl₂:methanol (2:1, v/v) and applied to the origin of a 0.5-mm Silica H plate along with standards of polar and neutral lipids. Plates were developed first 4 cm in diethyl ether, then fully in hexane:diethyl ether:acetic acid (70:30:1, v/v/v). The standard polar lipids (origin), MAGs (Rf 0.2), DAGs (Rf 0.4), FFAs (Rf 0.6), and TAGs (Rf 0.8) were resolved and located with light iodine staining. Radiolabeled lipid products were located using a Berthold LB 285 TLC Linear Analyzer (Berthold, Wildbad, FRG). Neutral lipids were scraped from the plate and eluted from the silica by extracting the latter three times with 3 mL water-saturated diethyl ether. The eluates were passed through 0.45-μm Cameo nylon filters (Micron Separations, Inc., Honeoye Falls, NY) and the solvent removed under nitrogen. The polar lipids were recovered by elution of the silica using the acidic Bligh and Dyer solution followed by phase separation as described above. The polar lipid fraction was re-chromatographed on 0.3-mm Silica H, first in CH₂Cl₂:methanol:ammonia (65:25:4, v/v/v), which resolved PC, PE, and their lyso-derivatives. The origin to Rf 0.2 region, containing PA and LPA, was scraped and the acidic phospholipids eluted using the acidic Bligh and Dyer procedure followed by phase separation. The LPA and PA were then resolved by TLC on 0.3-mm Silica H in CH₂Cl₂:methanol:acetic acid:water (100:40:12:4, v/v/v/v). Aliquots of the separated radiolabeled lipid species were quantified by counting on a LKB 1219 Rack-Beta instrument (LKB-Wallac Oy, Turku, Finland) in either 2.5 mL toluene plus 0.4% w/v 2,5-diphenyloxazole (New England Nuclear) for the neutral lipids, or in 2.5 mL Aquasol 2 (New England Nuclear) for the polar lipids.

When various acyl lipids were compared as acceptors for erucyl moieties, assays were conducted in the standard reaction mixture described above, except G-3-P was replaced by alternate acceptors, always at a final concentration of 200 μM. Before use in acceptor studies, aliquots of the sn-1,2-DAGs, purified as described earlier, were sampled, the hexane distilled off under a stream of nitrogen, and the DAG residues emulsified in 100 mM Hepes (pH 7.4) in the presence of 0.2% w/v Tween 20. In some cases, EDTA, DIPFP, and 2-BrO were tested as inhibitors of lipid biosynthesis under standard reaction conditions, at the final concentrations noted in the text.

Double Label Studies

All double label experiments were conducted under standard assay conditions except for the following modifications. In some cases, G-3-P was replaced in the standard reaction mixture by 500 μM 1-[2-¹³C]H(N)G-3-P (2.75 nCi⋅nmol⁻¹) and the biosynthesis of TAGs measured in the presence of 40 μM [1-¹³C]erucyl-CoA (6.8 nCi⋅nmol⁻¹). In addition, measurements of TAG biosynthesis were conducted in the presence of 200 μM dioleoyl-L-[2-¹³C]H(N)phosphatidylcholine (0.6 nCi⋅nmol⁻¹) and 40 μM [1-¹³C]erucyl-CoA, in both the presence and absence of unlabeled G-3-P (500 μM). The L-[2-¹³C]H(N)phosphatidylcholine (³H-PC) substrate was biosynthesized by incubating a homogenate prepared from 29-d late cotyledonary MD embryos (2.56 mg protein) with 10 μM L-[2-¹³C]H(N)G-3-P (10.6 Ci⋅mmol⁻¹), 40 μM 18:1-CoA, 1 mM MgCl₂, 0.5 mM ATP, 0.5 mM DTT, 0.5 mM EDTA, and 80 mM Hepes-NaOH, pH 7.4, in a final volume of 1 mL at 30°C with shaking (100 rpm). After 1 h, the reaction was supplemented with a second aliquot of 40 μM 18:1-CoA, and incubation continued for a further 60 min. The reaction was terminated and the total acyl lipids recovered as described above for the standard reaction mixture. The ³H-PC fraction was isolated by two-dimensional TLC on Silica H (0.5 mm) developed in CH₂Cl₂:MeOH:NH₄OH (65:25:4, v/v/v) in the first dimension, followed by CH₂Cl₂:acetone:MeOH:acetic acid:water (30:40:10:10:5 v/v/v/v/v) in the second dimension and located by co-chromatography with a dioleoyl-PC standard and confirmed using a radio-TLC linear analyzer. The ³H-PC fraction was scraped and recovered from the silica using the acidic Bligh and Dyer procedure and was then eluted with excess dioleoyl-PC to give a substrate that was >99% dioleoyl-PC with a final specific activity of 0.6 nCi⋅nmol⁻¹.
Further Characterization of Radiolabeled TAGs

Intact radiolabeled TAGs were separated by HPLC. The system was composed of an SP7800 liquid chromatograph (Spectra Physics, San Jose, CA) furnished with an ACS model 750/14 Evaporative Analyzer (Applied Chromatography Systems Ltd., Macclesfield, Cheshire, UK) and a Flo-One/Beta radioactive flow detector and data processing program (Radiomatic Instruments and Chemical Co., Tampa, FL). The HPLC columns consisted of a Whatman PartiSphere C18 5-μm reverse-phase cartridges (4.6 mm i.d. x 12.5 cm) (Whatman Inc., Clifton, NJ) in series. The two mobile phase solvents were acetone and acetonitrile. The TAGs were resolved with the following gradient: 50% acetone/50% acetonitrile at time 0, to 60% acetone/40% acetonitrile at 4 min, to 65% acetone/35% acetonitrile at 20 min, to 100% acetone at 30 min. Following a 5-min isocratic period at 100% acetone, the column was re-equilibrated to 50% acetone/50% acetonitrile over 5 min. The HPLC flow rate was 2 mL·min⁻¹. The column eluate was split, with 10 to 20% going to the evaporative analyzer and the remainder going to the radioactive flow detector. Before entering the latter, the column effluent was mixed with scintillant (toluene plus 0.4% w/v 2,5-diphenyloxazole) at a rate of 2 mL·min⁻¹. Radiolabeled TAGs were identified by co-chromatography with external TAG standards detected with the evaporative analyzer, and were confirmed by direct probe MS of HPLC fractions.

Argentation-TLC of radiolabeled TAGs was performed on Silica Gel G impregnated with 10% (w/v) AgNO₃ (16) in chloroform:MeOH (99:1, v/v). After locating radiolabeled spots on the TLC using the Berthold LB 285 Linear Analyzer, the TAG fractions were eluted and aliquots were characterized by GC-MS. Positional analyses were performed on radiolabeled TAGs by treatment with pancreatic lipase (16) and a modified Brockerhoff stereospecific analysis essentially as described by Christie (16).

Test of Insertion of Erucoyl Moieties by a Postsynthetic Modification of Preformed TAGs

Reactions were conducted in mixtures containing 80 mM Hepes-KOH (pH 7.4), 1 mM ATP, 0.3 mM CoASH, 2 mM MgCl₂, and 0.2 to 0.5 mg homogenate protein. The following acyl donor plus TAG acceptor combinations were tested, with all TAGs being emulsified in 0.5% (w/v) gum arabic: 40 μM erucoyl-CoA plus 200 μM [1-¹⁴C]triolein (10 nCi·nmol⁻¹); 40 μM [1-¹⁴C]erucoyl-CoA (10 nCi·nmol⁻¹) plus 200 μM triolein; 1 mM [2-¹³C]malonyl-CoA (10 nCi·nmol⁻¹) plus 200 μM triolein; 40 μM [1-¹³C]erucoyl-CoA (10 nCi·nmol⁻¹) plus 200 μM 1,2-dioleyl,3-erucoylglycerol; 1 mM [2-¹³C]malonyl-CoA (10 nCi·nmol⁻¹) plus 200 μM 1,2-dioleyl,3-erucoylglycerol. After 2 to 6 h at 30°C, 100 rpm, reactions were stopped, phases separated, the TAG fraction isolated by TLC and intact TAGs analyzed by radio-HPLC as described above.

RESULTS AND DISCUSSION

Comparison of Acyl Composition of Total Lipids and TAG Accumulation in Developing Microspore-Derived and Zygotic Embryos

The fatty acid composition of total acyl lipids isolated from developing MD Reston embryos showed a strong similarity

to that observed in developing zygotic seeds of the same cv, particularly with respect to accumulation of the 18:1, 20:1, and 22:1 acyl moieties (Fig. 1). In both MD and zygotic embryos, the mol % proportions of cis-9-eicosenoic and erucic acids increased from 2 to 3% and 1%, respectively, at early cotyledonary stages, to 9 to 13% and 12 to 15%, respectively, by late cotyledonary stages. In both embryo types, the proportion of 18:1 fell concomitantly with an initial rise in proportions of 20:1 followed by 22:1 during the later cotyledonary stages of embryogenesis. Such trends suggest that in MD embryos, 20:1 and then 22:1, are biosynthesized from oleoyl-CoA via successive condensations with malonyl-CoA, as is known for developing zygotic embryos of B. napus (43), Brassica juncea (1), and other oilseeds (3, 28, 31, 34, 35). Other major acyl groups present throughout embryogenesis in both microspore-derived and zygotic systems (data not shown) were 18:2, 18:3, and 16:0. The mol % proportions of these three fatty acids decreased from heart stage (18:2 = 31-32%, 18:3 = 14-18%, 16:0 = 8-17%) to very late cotyledonary stage (18:2 = 16-18%, 18:3 = 9-10%, 16:0 = 4%). Freshly isolated Reston microspores had about 48% 18:3, 16% 18:2, and 15% 16:0, in addition to 18:1.

When compared on a dry weight basis, the acyl composition of lipids from very late cotyledonary stage MD embryos was similar to that of mature zygotic seed (Table I). Total fatty acids comprised about 45% of the dry weight in both embryos.

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**Figure 1.** Changes in fatty acyl composition of a total lipid extract with respect to proportions of 18:1, 20:1, and 22:1 found in microspore-derived and zygotic embryos of B. napus L. cv Reston at different stages of development. The developmental stages designated are further defined in terms of d in culture or d postanthesis in "Materials and Methods." The 20:1 and 22:1 were not detected before the torpedo stage in either embryo system.
Table I. Fatty Acid Levels in Total Acyl Lipids of B. napus cv Reston Seed and Very Late Cotyledonary Stage MD Embryos

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Zygotic seed</th>
<th>35-d MD embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg·g dry wt⁻¹⁻¹b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>18:0</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>18:1</td>
<td>95</td>
<td>173</td>
</tr>
<tr>
<td>18:2</td>
<td>62</td>
<td>55</td>
</tr>
<tr>
<td>18:3</td>
<td>34</td>
<td>31</td>
</tr>
<tr>
<td>20:1</td>
<td>54</td>
<td>70</td>
</tr>
<tr>
<td>22:1</td>
<td>168</td>
<td>138</td>
</tr>
<tr>
<td>Total</td>
<td>435</td>
<td>488</td>
</tr>
</tbody>
</table>

* Small amounts of 20:0 and 22:0 (<2 mg·g dry wt⁻¹) were also present.  
* Calculated from fresh weight using conversion values of 95% (zygotic seed) and 40% (MD embryos) for dry weight expressed as a percentage of fresh weight.

Although differences in the levels of 18:1 and 22:1 were observed between the MD embryos and the zygotic seed. Such differences may be due to the relatively slow accumulation of 22:1 during the late cotyledonary stages of MD embryogenesis. In a previous study, it was demonstrated that in B. napus cv Topas, a cv that does not accumulate 22:1, the lipid composition of very late-cotyledonary stage MD embryos was virtually identical to that of the dry seed (50).

In vivo rates of TAG biosynthesis, estimated from measurements of TAG, dry weight, and protein during the rapid phase of lipid accumulation, were compared in developing Reston zygotic embryos (4–8 weeks after anthesis) and MD embryos (2–5 weeks in culture). Zygotic and MD embryos accumulated TAGs at rates of 10.0 and 10.7 µg·min⁻¹·mg dry weight⁻¹, and 75 and 142 pmol·min⁻¹·mg protein⁻¹, respectively. Thus, the rates were essentially identical when expressed on the basis of dry weight. While of the same order of magnitude, the difference in rates of TAG accumulation expressed on a mg protein basis may be due to inherent differences between zygotic and MD embryos in the accumulation of storage proteins (e.g. cruciferin) as reported previously (50).

Further Analysis of Endogenous Acyl Lipid Species in Developing MD Embryos

Analyses of the fatty acid composition of acyl lipids in late cotyledonary stage MD embryos indicated the virtual absence of 22:1, but the presence of significant 20:1, in polar lipids and MAGs (Table II). However, both erucyl and eicosenoyl moieties were detected in comparable proportions in the TAGs, DAGs, and FFA s. The inability to detect 22:1 in the MD embryo polar lipid fraction agrees with comparable studies of zygotic embryos. In general, 22:1 does not accumulate in the phospholipid or membrane fractions of developing oilseeds, but is found almost exclusively in the neutral lipid fraction (2, 23, 29, 34, 38). Eicosenoic acid has been reported to be detected in the PC fraction of Brassica campestris (22).

GC/EI-MS analyses of trimethylsilyl derivatives of endogenous intact DAGs isolated from MD embryos (data not shown) indicated, among others, the presence of the DAG species 20:1/18:1 (fragment ions [M - 15]+ = 705; [M - OTMS]+ = 630; [M - RCO₂]+ = 439, 411) and 22:1/18:1 (fragment ions [M - 15]+ = 733; [M - OTMS]+ = 658; [M - RCO₂]+ = 467, 411). In general, the GC/MS data were consistent with the total acyl composition of the DAG fraction reported in Table II, with C₁₈ DAGs predominating.

Intact TAGs were analyzed by direct probe EI-MS (Fig. 2). Major molecular ion clusters were observed for monoeicosenoyl (M⁺ = 909–913), monoeicosenoyl (M⁺ = 937–941), monoeicosenoyl, monoeicosanylenoyl (M⁺ = 965–969), and diaracylic (M⁺ = 993–997) TAG species (Fig. 2A). Each molecular ion cluster represents TAGs containing combinations of these acyl groups with 18:1, 18:2, or 18:3 on the glycerol backbone. A prominent molecular ion was observed for trilinolenyl (M⁺ = 885) and the cluster of ions in this region (877–885) represented combinations of C₁₂ fatty acyl moieties in the TAGs. Trierucin (M⁺ = 1053) was not found. Interestingly, a small cluster at M⁺ = 1025 corresponding to a diaracylic, monoeicosanylenoyl-glycerol was evident. Characteristic TAG fragmentation ions were observed in the [M - 18]⁺ region (Fig. 2A), [M - RCO₂H]⁺ region (data not shown), and in the [RCO + 74]⁺, [RCO + 115]⁺, and [RCO + 128]⁺ regions (Fig. 2B) (5, 17).

Structural assignments were confirmed by MS/MS daughter ion analyses (mass spectra not shown). For example, the parent molecular ion M⁺ = 969, assigned the structure erucoyl, oleoyl, eicosanylenoyl-glycerol, was found to have [M - RCO₂H]⁺ daughter fragment at m/z = 687, 631, and 659 and [RCO + 74]⁺ fragments at m/z = 395, 339, and 367. The

Table II. Fatty Acyl Composition of Endogenous Lipid Classes Isolated from Late Cotyledonary Stage (28 d) MD Embryos of B. napus cv Reston

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
<th>20:0</th>
<th>20:1</th>
<th>22:0</th>
<th>22:1</th>
<th>% dp⁻¹⁺⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polar lipid</td>
<td>8.9</td>
<td>4.7</td>
<td>50.5</td>
<td>22.0</td>
<td>9.8</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>ND</td>
<td>98.9</td>
</tr>
<tr>
<td>MAG</td>
<td>30.6</td>
<td>22.7</td>
<td>32.6</td>
<td>9.5</td>
<td>tr</td>
<td>tr</td>
<td>4.7</td>
<td>ND</td>
<td>ND</td>
<td>99.2</td>
</tr>
<tr>
<td>DAG</td>
<td>6.3</td>
<td>2.8</td>
<td>62.8</td>
<td>15.4</td>
<td>3.9</td>
<td>0.6</td>
<td>4.8</td>
<td>0.4</td>
<td>3.0</td>
<td>99.8</td>
</tr>
<tr>
<td>FFA</td>
<td>9.7</td>
<td>7.0</td>
<td>53.5</td>
<td>8.3</td>
<td>2.0</td>
<td>2.0</td>
<td>7.5</td>
<td>1.5</td>
<td>8.5</td>
<td>99.7</td>
</tr>
<tr>
<td>TAG</td>
<td>5.6</td>
<td>3.1</td>
<td>54.5</td>
<td>12.1</td>
<td>4.7</td>
<td>1.1</td>
<td>8.0</td>
<td>0.5</td>
<td>8.0</td>
<td>97.2</td>
</tr>
</tbody>
</table>

* Percent of detectable peaks on gas chromatogram. * tr, Trace (<0.2%).  
* ND, Not detected.
creatic
lipase,
extent
found
are
to
32).
and
20:1
tag
dierucoyl
little
as
514
TAG
dierucoyl
are
parent
(Rco)
group:
mercial
[M
18]+
fragmentation
indicated
by
arrows
from
each
parent
ion.
B.
[RCO
+74]],[RCO
+115]+,[RCO
+128]+
region.
RCO
+74]+
fragment
ions
are
designated
for
the
acyloxy
(Rco)
group:
a
= C18H33O2,
b
= C20H37O,
c
= C19H34O.
the
RCO
+115]+
and
RCO
+128]+
fragment
ions
are
designated
by
the
same
letter.

parent
molecular
M
= 997,
assigned
the
structure
EOE
was
found
to
have
[M
RCO2H]+
daughter
fragments
at
m/z
659
and
716
and
RCO
+74]+
fragments
at
m/z
395
and
339.
It
was
interesting
and
perhaps
surprising,
that
with
as
little
as
8.5
mol
% 22:1
in
the
entire
TAG
fraction,
dierucoyl
TAG
species
were
detected.
Such
findings
dem-
strate
the
value
of
MS
as
a
technique
allowing
intact
TAGs
to
be
identified.
Furthermore,
one
is
reminiscent
of
the
limitations
in
inferring
probable
TAG
species
based
solely
on
acyl
compositions
acquired
via
transmethylation
analyses.

In
zygotic
B.
apus
varieties
containing
a
high
content
of
20:1
and
22:1,
it
has
been
reported
that
these
fatty
acyl
groups
are
found
in
the
sn-3
position
of
TAGs,
slightly
less
extensive
in
the
sn-1
position,
but
not
at
the
sn-2
position
(8,11,
32).
Treatment
of
endogenous
MD
embryo
TAGs
with
pancreatic
lipase,
followed
by
transmethylation
of
the
FFAs
released
from
the
sn-1
and
sn-3
positions
and
the
remaining
sn-2
acyl
MAGs,
confirmed
that
erucoyl
moieties
were
present
at
the
sn-1
+3
positions
but
absent
at
the
sn-2
position.
This
is
supported
by
the
data
from
TAG
analyses
by
MS,
which
indicated
that
trierucin
was
not
found
in
MD
embryos
of
B.
apus
cv
Reston.
However,
the
presence
of
a
detectable
level
of
dierucoyl,
monoeicosoenyl
TAG
in
the
EI-mass
spectrum
of
endogenous
TAGs
(M0 = 1025;
Fig.
2A)
suggests
that,
least
in
the
MD
embryo
system,
20:1
moieties
are
not
absolutely
excluded
from
the
sn-2
position
of
TAGs.

From
the
analyses
of
the
endogenous
acyl
lips
of
developing
MD
embryos,
it
was
apparent
that
at
the
mid-late
cotyledonary
stage,
20:1
and
22:1
were
synthesized
and
accumulated
in
the
neutral
lipid
fraction
and
that
the
enzymatic
machinery
was
in
place
for
producing
DAGs
containing
erucoyl
moieties
at
the
sn-1
position
and
TAGs
containing
22:1
at
both
the
sn-1
and
sn-3
positions.

Comparison
of
In
Vitro
TAG
Bioassembly
in
MD
versus
Zygotic
Embryo
Systems

Initial
studies
conducted
utilizing
homogenates
and
microsomal
fractions
prepared
from
late
(29-d)
cotyledonary
stage
MD
embryos
had
indicated
that,
in
the
presence
of
G-3-P,
radionabeled
erucoyl
moieties
were
very
rapidly
incorporated
into
the
TAG
fraction,
but
the
mechanism
was
not
reported
(50).
In
the
present
study,
MD
and
zygotic
embryos
were
compared
to
see
whether
they
could
synthesize
TAGs
in
vivo,
utilizing
radionabeled
erucyl-CoA
as
an
acyl
donor
and
G-3-
P
as
an
acceptor,
that
could
support
those
estimated
for
TAG
accumulation
in
vivo.
While
the
zygotic
embryo
system
incorporated
erucoyl
moieties
poorly
into
TAGs
in
vivo,
both
homogenates
and
microsomal
fractions
prepared
from
the
MD
embryo
system
were
highly
active
in
this
regard,
exhibiting
rates
that
could
easily
account
for
those
observed
in
vivo
(Table
III).
The
high
rates
of
TAG
biosynthesis
observed
in
the
presence
of
G-3-P
and
14C
22:1-CoA
in
vivo
in
the
MD
embryo
system
also
exceeded
those
reported
in
comparable
studies
utilizing
homogenates
or
microsomal
fractions
prepared
from
various
developing
zygotic
embryos,
including
B.
apus
(45),
B.
juncea
(22),
and
Crambe
abyssinica
(7),
by
30- to
50-fold.
Given
such
a
high
capacity
to
incorporate
erucoyl
moieties
into
storage
lips,
it
was
decided
to
exploit
the
MD
embryo
system
to
find
studies
of
TAG
bioassembly
in
the
Brassicaceae.

In
Vitro
Studies
of
Lipid
Biosynthesis
in
the
MD
Embryo
System:
Metabolism
of
Oleoyl
versus
Erucoyl
Moieties

Initially,
the
pathways
by
which
oleoyl
and
erucoyl
moieties
were
incorporated
into
acyl
lips
were
compared.
In
the
presence
of
G-3-P
and
14C
18:1-CoA,
homogenates
of
29-d,
mid-late
cotyledonary
stage
MD
embryos
were
able
to
rapidly
incorporate
radionabeled
18:1
into
PA
and
a
to
lesser
extent,
lysop-PA,
DAG,
and
TAG,
as
well
as
PC,
and
other
complex
polar
lipids
(Fig. 3A).
These
data
are
consistent
with
oleoyl
incorporation
into
TAGs
via
the
Kennedy
Pathway,
as
well
as
into
membrane
lipid
components
(e.g.
PCs,
PEs),
as
is
known
for
zygotic
oilseeds
(7,28,30,44,45).
In
particular,
the
rates
of
incorporation
of
18:1
moieties
into
LPA,
PA,
and
DAG were in the general range reported in studies of oilseeds of various *B. napus* cvs (8, 30, 45). Transmethylation of the radiolabeled acyl lipid species (data not shown) showed primarily the presence of \(^{14}C\)-18:1, and to a lesser extent, \(^{14}C\)-18:2 and -18:3 moieties. There was no evidence of \(^{14}C\)-20:1 or -22:1, indicating that, while \(^{14}C\)-18:1 moieties were able to undergo desaturation, the elongation of \(^{14}C\)-18:1-CoA was not observed *in vitro* in the absence of exogenously added malonyl-CoA. Similar findings have been reported in studies of oleoyl elongation conducted using zygotic embryos (1, 31, 43).

In contrast, when parallel experiments were conducted in the presence of G-3-P and \(^{14}C\)-22:1-CoA, radiolabeled erucoyl moieties were incorporated only into TAGs and the FFA pool. \(^{14}C\)-22:1 moieties did not accumulate in Kennedy pathway intermediates (lyso-PA, PA, or DAG) or in PC, PE, or their lyso-derivatives (Fig. 3B), or other complex polar lipids such as phosphatidylinositol, phosphatidylserine, monogalactosyldiacylglycerol, and digalactosyldiacylglycerol (data not shown). This pattern was unchanged during time course experiments. In as little as 5 min, radiolabeled erucoyl moieties were either rapidly and exclusively incorporated into TAGs or released as FFA. The incorporation of \([1\text{-}{^{14}}C]22:1\text{-CoA}\) into TAGs was linear over 30 min with up to 1 mg of homogenate protein and was optimal in the presence of 200 to 500 \(\mu M\) G-3-P and 40 \(\mu M\) erucoyl-CoA, in general agreement with previous findings (50). Analyses of the radiolabeled TAGs with pancreatic lipase indicated that >95% of the \(^{14}C\)-22:1 was esterified to the sn-1 + 3 positions. This pattern of metabolism is consistent with the exclusion of erucoyl moieties from the sn-2 position of endogenous TAGs as discussed above. The inability to incorporate erucoyl moieties *in vitro* into LPA, PA, and DAG or membrane lipid components when G-3-P is supplied as the acceptor, is consistent with previous studies of erucoyl-CoA metabolism conducted in oilseeds (4, 7, 45). Particulate fractions from the zygotic embryos of *B. napus* high erucic acid cvs Reston and Golden were unable to insert erucoyl moieties into the sn-1 position of G-3-P (DC Taylor, unpublished observations). However, recently, Bernerth and Frenzten (8) reported that erucoyl-CoA was incorporated to some extent into the sn-1 position of G-3-P by a 20,000g particulate fraction from developing seed of *B. napus* L. cv Lenora.

The appreciable level of radiolabeled 22:1 found in the FFA fraction was presumably due to the action of a 22:1-CoA:thioesterase present in the MD embryo homogenate. Erucoyl-CoA:thioesterase activity has been reported in homogenates of late cotyledonary MD embryos (50). However, it was also found that, in the presence of ATP and CoASH, embryo homogenates converted \([1\text{-}{^{14}}C]22:1\text{FFA}\) to \([1\text{-}{^{14}}C]22:1\text{-CoA}\), via an endogenous 22:1-CoA synthetase. The newly synthesized erucoyl-CoA was subsequently incorporated into TAGs, although there was a lag phase compared with when \([1\text{-}{^{14}}C]22:1\text{-CoA}\) was supplied directly. This was consistent with the need to synthesize the 22:1-CoA thioester as a prerequisite for incorporation of erucoyl moieties into acyl lipids.

### Inhibitor Studies

It has been reported that EDTA inhibits phosphatidate phosphatase (EC 3.1.3.4) in microsomal fractions from developing safflower (44), whereas DIPFP and 2-BrO have been shown to inhibit DAG acyltransferase (EC 2.3.1.20) activities

[Table III. Comparison of in vivo and in vitro Rates of TAG Biosynthesis in Developing Zygotic and MD Embryos of *B. napus* cv Reston]

<table>
<thead>
<tr>
<th>Embryo System</th>
<th>Rate of TAG Biosynthesis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>in vivo</em> (^{a})</td>
<td><em>in vitro</em> (^{b})</td>
</tr>
<tr>
<td></td>
<td>Homogenate</td>
<td>Microsomes</td>
</tr>
<tr>
<td>Zygotic</td>
<td>75</td>
<td>6 (8%) (^{c})</td>
</tr>
<tr>
<td>Microspore-derived</td>
<td>142</td>
<td>166 (117%)</td>
</tr>
</tbody>
</table>

\(^{a}\) Estimated from measurements of TAG, total protein, and dry weight in developing mid-late cotyledonary stage embryos during the rapid phase of TAG accumulation: zygotic embryos, 4 to 8 weeks postanthesis; MD embryos, 2 to 5 weeks in culture. \(^{b}\) Measured in homogenates or microsomal preparations from mid-late cotyledonary stage embryos using the reaction system: G-3-P plus \(^{14}C\)-22:1-CoA, and assuming one erucoyl moiety incorporated per TAG (18/1:18:1/\(^{14}C\)-22:1) synthesized. \(^{c}\) Values in parentheses express the *in vitro* rate as a percentage of the estimated *in vivo* rate.

[Figure 3. Comparison of glycerolipids synthesized by a homogenate prepared from late cotyledonary stage MD embryos of *B. napus* cv Reston, in the presence of 200 \(\mu M\) G-3-P and either (A) 40 \(\mu M\) \(^{14}C\)-18:1-CoA or (B) 40 \(\mu M\) \(^{14}C\)-22:1-CoA.]

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in developing safflower (25) and cultured rat hepatocytes (15, 26), respectively. Thus, these chemicals were tested for their effects on the incorporation of radiolabeled erucoyl-CoA into acyl lipids in the MD embryo system. In the presence of G-3-P as acyl acceptor, 25 mM EDTA, 5 mM DIPFP, and 1.5 mM 2-BrO decreased the rate of incorporation of 14C-22:1 moieties into TAGs by 85%, 60%, and 80%, respectively, but in no case was the inhibition accompanied by an accumulation of 14C-22:1 in hysO-PA, PA, DAG or other complex polar lipids. In contrast, a parallel experiment conducted utilizing 14C-18:1-CoA in the presence of 2-BrO indicated that levels of 14C-18:1 in LPA/PA and DAG increased by 170% and 70%, respectively, whereas 14C-18:1 in TAGs decreased by 70%. Thus, the inhibitor data support the incorporation of oleoyl-CoA, but not erucoyl-CoA, into LPA, PA, and DAG when G-3-P is supplied as substrate.

Acceptors Studies and Analyses of Radiolabeled TAGs

G-3-P and a number of acyl lipids were tested as acceptors to probe the mechanism by which 14C-22:1 was incorporated into TAGs. With the exception of DHAP, all substrates screened gave incorporation rates greater than that observed in the absence of exogenous acceptor (Fig. 4). G-3-P, LPAs, and DAGs increased the basal rate of [14C]TAG biosynthesis by six- to 10-fold. However, the use of LPA or PA as acceptors did not yield the expected radiolabeled PA or DAG products. Regardless of the substrate supplied, only the TAG fraction became radiolabeled. HPLC analyses of the radiolabeled TAGs produced in the presence of each acceptor indicated an interesting phenomenon: irrespective of the acyl acceptor provided, only the four monoerucoyl radiolabeled TAG species LnOE, LOE, OOE, and POE were formed in relative proportions of 12%, 30%, 55%, and 3%, respectively (Fig. 5). Radiolabeled dieucroyl TAG species were not detected. Even sn-1 22:1-LPA and sn-1,2 22:1-PA failed to yield di- or trierucoyl [14C]TAGs, yet greatly enhanced 14C-22:1 incorporation over rates observed in the absence of acceptor. It should be noted that stereospecificity of nonradiolabeled acyl moieties depicted at the sn-1 and sn-2 positions in most Figures

and Tables were not determined and are not absolute. These short-form designations are for the purposes of discussion and indicate only that these acyl moieties were present in the intact TAGs. Regardless of the actual stereospecific composition, TAGs containing identical acyl compositions (e.g. 18:2/18:1/22:1, 18:1/18:2/22:1) were not resolved by reverse-phase HPLC. Argetnation TLC of radiolabeled TAGs (Fig. 6) followed by elution and GC-MS of radiolabeled fractions confirmed that, in the presence of G-3-P and 14C-22:1-CoA, only the four monoerucoyl [14C]TAGs described above were produced in vitro.

Analyses of radiolabeled TAGs from 14C-18:1-CoA incorporation experiments revealed species containing C18 moieties, the primary 14C products being LOL, LOO, and OOO (Fig. 7). There was also some evidence for a small amount of LnOE, but this was not clearly resolved from OOO by the HPLC gradient. Transmethylation of the total radiolabeled TAG fraction showed a 14C-fatty acid distribution of 76% 14C-18:1, 21% 14C-18:2, and 3% 14C-18:3. There was no evidence of TAGs containing radiolabeled 20:1 or 22:1 moieties, potential products of 14C-18:1-CoA elongation, for reasons discussed above.

Brockerhoff stereospecific analyses (11) of the [14C]TAGs produced in experiments in which embryo homogenates were
supplied with G-3-P and either 14C-22:1-CoA or 14C-18:1-CoA, indicated that the radiolabeled erucyl moieties were incorporated exclusively into the sn-3 position, while the oleoyl moieties were restricted to the sn-1 and sn-2 positions (Table IV). Given the number of complex hydrolytic and synthetic steps involved in a stereospecific analysis (16), the very small proportion of 14C-22:1 detected at the sn-1 position was well within the range normally attributed to limited acyl migration during product isolation at almost any stage of the method. The data suggested that, while all three acyltransferases of the Kennedy Pathway leading from G-3-P to TAGs (44) were operating in the MD embryo homogenate system, there was some degree of acyl-CoA specificity: erucyl groups were inserted by DGAT into the sn-3 position, while oleoyl moieties were incorporated via G-3-PAT and LPAT, into the sn-1 and sn-2 positions, respectively. The same stereospecific 14C-22:1 distribution pattern was confirmed in a microsomal protein fraction. Furthermore, a homogenate from early (14-d) cotyledonary embryos gave similar results (Table IV), indicating that such positional selectivity was not dependent upon the stage of embryo development. The inability to detect radiolabeled oleoyl moieties on the sn-3 position of TAGs synthesized in the presence of G-3-P may indicate the high affinity for oleoyl-CoA exhibited by G-3-PAT and LPAT relative to DGAT, in late-cotyledonary stage MD embryos.

**Erucyl-CoA:DGAT Activity**

The cumulative data suggested that when 14C-22:1-CoA was the substrate, erucyl moieties were incorporated exclusively via a DGAT reaction. When sn-1,2-dierucin was supplied as an acyl acceptor, in addition to the monerucyl TAG species described above, the nonindigenous TAG trierucin was also produced, comprising about 25 to 35% of the radiolabeled TAG product (Fig. 4). The presence of trierucin

![Figure 6](image_url)

**Figure 6.** Argentation TLC scan of radiolabeled TAGs formed by a homogenate of late cotyledonary stage MD embryos of B. napus cv Reston incubated in the presence of 200 μM G-3-P and 40 μM 14C-22:1-CoA. TAG acyl compositions are reported with numbers indicating degrees of unsaturation (0 = saturated, 1 = monoenoic, 2 = dioenoic, 3 = trienoic fatty acyl moieties) and indicating a 14C-labeled acyl group. Probable degrees of unsaturation were assigned based on migration of synthetic TAG standards on the same TLC plate. Following elution from the silica, GC-MS analyses of the radiolabeled TAG fractions revealed the same four monoerucyl TAG species identified by HPLC analysis (Fig. 5).

![Figure 7](image_url)

**Figure 7.** Reverse-phase HPLC analysis of radiolabeled TAGs formed by a homogenate of late cotyledonary stage MD embryos of B. napus cv Reston incubated in the presence of 200 μM G-3-P and 40 μM 14C-18:1-CoA. The radiolabeled TAG sample was split with 10% going to a mass detector (evaporative analyzer; top panel) and the remaining 90% going to the radioactive flow detector (bottom panel). TAG species were identified by co-chromatography with external TAG standards, located using the mass detector. Abbreviations: LOE, LnOE, etc.: TAG species composed of erucyl (E), eicosenoyl (Ei), linoleoyl (L), linolenoyl (Ln), oleoyl (O), and palmitoyl (P) moieties.

---

**Table IV. Stereospecific Distribution of Radioactive Acyl Moieties in TAGs**

Homogenates or microsomal fractions prepared from developing MD embryos of B. napus cv Reston at early (14 d) or late (29 d) cotyledonary stages were incubated with G-3-P and either 14C-18:1-CoA or 14C-22:1-CoA. The radiolabeled TAG fraction was isolated as described in "Materials and Methods," and stereospecific analyses performed by a modified Brockerhoff approach (16).

<table>
<thead>
<tr>
<th>Fraction/Embryo Age</th>
<th>14C-Acyl-CoA Supplied</th>
<th>Distribution of Radioactivity at Glycerol Backbone Position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>sn-1</td>
</tr>
<tr>
<td>Homogenate/29 d</td>
<td>18:1</td>
<td>49</td>
</tr>
<tr>
<td>Homogenate/29 d</td>
<td>22:1</td>
<td>tr*</td>
</tr>
<tr>
<td>Homogenate/14 d</td>
<td>22:1</td>
<td>4</td>
</tr>
<tr>
<td>Microsomes/14 d</td>
<td>22:1</td>
<td>3</td>
</tr>
</tbody>
</table>

* tr = Trace (<1%).
was confirmed by radio-HPLC and by direct probe EI-MS of the radiolabeled TAG fraction, which showed a molecular ion at m/z = 1053 (data not shown).

There is current interest in genetic modification of rapeseed to produce seed oils very high in 22:1, by introducing into B. napus a foreign LPAT that is capable of inserting erucoyl moieties at the sn-2 position. Recent studies in this laboratory (47) and others (14) have shown that whereas B. napus is essentially devoid of an active erucoyl-CoA:LPAT, Meadowfoam species (Limnanthes douglasii [47] and Limnanthes alba [14]) possess a highly specific erucoyl-CoA:LPAT. Thus, the meadowfoam LPAT would appear to be an excellent candidate for supplying a target gene to genetically engineer rapeseed to produce trierucin. However, the final success of such a program would depend on whether, for example, B. napus DGAT is capable of acting on dierucoyl-DAG. The metabolic studies reported here, where [14C]trierucin is produced in the presence of sn-1,2-dierucin and 14C-22:1-CoA, would suggest that the B. napus DGAT is able to catalyze such a conversion.

When sn-1,2-dierucin or -diolein was tested as acceptor in the presence of 2-BrO, incorporation of 14C-22:1 into TAGs was inhibited by 70% to 80% (Table V). To our knowledge, this is the first report of the use of this chemical to inhibit DGAT activity in oilseed metabolism studies, although its effects have been documented in animal systems (15, 26). It has been suggested that the inhibition of DGAT by 2-BrO in mammalian systems is, in fact, accomplished by the corresponding 2-BrO-CoA thioester, synthesized in vitro from 2-BrO via a mitochondrial acyl-CoA synthetase (26). While we have shown that a long-chain acyl-CoA synthetase activity is present in the B. napus MD embryo system (50), it is not yet clear whether 2-BrO or its CoA thioester is the active inhibitor of the DGAT in this system. Nevertheless, 2-BrO-CoA may prove to be a more potent inhibitor of DGAT in vitro, and we are investigating this possibility.

The specific activity of DGAT expressed in homogenates varied depending on the acceptor substrate supplied (Table V). In addition, the relative rates observed between G-3-P and DAG acceptors differed somewhat depending on whether homogenates were prepared from early (Table V) or late (Fig. 4) cotyledonary stages.

In localization studies performed in late cotyledonary stage MD embryos using differential centrifugation, DGAT activity was found in oil body and microsomal fractions, but was essentially absent from the soluble (105,000g supernatant) fraction (Fig. 8). A small amount of activity was also initially detected in the 10,000g pellet (data not shown), but after two rounds of washing in grinding medium followed by re-centrifugation at 10,000g to re-precipitate the protein, DGAT activity was lost from the pellet fraction. Thus the activity initially measured in this fraction was probably due to contamination from the microsomal fraction. The absence of significant DGAT activity in the soluble fraction was consistent with data reported for a similar fraction from zygotic B. napus, where 18:1-CoA was the acyl donor supplied (30). In the present study, about 65% of the total activity and about 7% of the total protein were recovered in the microsomal pellet, and this fraction yielded the highest specific activity of DGAT. The absolute activity expressed in this fraction was greater when G-3-P, rather than DAG, was supplied as acceptor (Table V). In general, the activities recorded in the presence of DAG acceptors in this study were comparable to 22:1-CoA:DGAT activities recorded in microsomal preparations from zygotic embryos of high 22:1 cv of B. napus (8, 13).

However, the rate of incorporation of 14C-erucoyl moieties into TAGs in the presence of G-3-P was much greater in the MD embryo system than had been observed in parallel

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**Table V. Effects of Exogenously Supplied Acceptors and 2-Bromooctanoate on DAG Acyltransferase Activity in Homogenate and Microsomal Fractions from Developing Cotyledonary MD Embryos of B. napus cv Reston**

<table>
<thead>
<tr>
<th>Reaction Conditions</th>
<th>Homogenate</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ G-3-P</td>
<td>100</td>
<td>300</td>
</tr>
<tr>
<td>+ G-3-P + 2-BrO</td>
<td>30 (70)</td>
<td>—</td>
</tr>
<tr>
<td>+ 18:1 DAG</td>
<td>55</td>
<td>114</td>
</tr>
<tr>
<td>+ 18:1 DAG + 2-BrO</td>
<td>11 (80)</td>
<td>—</td>
</tr>
<tr>
<td>+ 22:1 DAG</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>+ 22:1 DAG + 2-BrO</td>
<td>13 (68)</td>
<td>—</td>
</tr>
</tbody>
</table>

*14C-22:1-CoA was the acyl donor in all cases. Stereospecific analyses indicated that 14C-22:1 was incorporated exclusively into the sn-3 position. Values in parentheses show percent inhibition of activity relative to the same reaction performed without 2-BrO. —, Not determined.

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![Figure 8. DAG acyltransferase activity present in subcellular fractions obtained by differential centrifugation. Incorporation of 14C-22:1 moieties into the sn-3 position of TAGs was monitored in the presence (closed triangle) and absence (open triangle) of 200 μM G-3-P.](image-url)
vitro studies of zygotic embryo metabolism, as discussed above. In the MD embryo TAGs, 14C-erucoyl moieties were incorporated only into the sn-3 position. Furthermore, this was accomplished without supplying other exogenous acyl-CoA species to act as acyl donors for the sn-1 and sn-2 positions on G-3-P. Thus, it was somewhat surprising that the specific activity of the microsomal 22:1-CoA:DGAT was higher when assayed in the presence of G-3-P than when assayed in the presence of any DAG species, (Table V), given that the latter would be expected to be the more direct substrate. In addition, as previously discussed, all acyl acceptors tested gave 14C-22:1 TAG incorporation patterns identical to those observed with G-3-P (Fig. 5). These findings suggested that, rather than acting as direct acceptors, some of the substrates tested might be acting as activators or modulators of one or more Kennedy pathway enzymes, thus affecting the size of the DAG pool, with the latter being the true acceptor of 14C-erucoyl moieties.

One mechanism for controlling overall DAG levels during TAG biosynthesis might be to regulate the conversion of PCs to DAGs via the enzyme CPTase. Slack, Roughan et al. (38, 40, 41) were the first to demonstrate that this enzyme existed in developing oilseeds. Subsequently, it was shown that safflower microsomes would catalyze the simultaneous movement of the DAG backbone into PC and the removal of glycerol moieties from PC to DAG during the synthesis of TAGs via the Kennedy pathway; in short, the enzyme could operate in a reversible fashion (42). Furthermore, CDP-choline and CMP, the cofactors of the forward and reverse reactions of CPTase, respectively, were not found to be rate-limiting in microsomal preparations of safflower (42).

Double Label Studies

Initially, double label experiments were conducted to assess whether \( \text{L}-(2-^{3}H) \text{N}) \text{G-3-P} \) was acting as a true acceptor of 14C-erucoyl moieties in the biosynthesis of TAGs. \(^{3}H\) from exogenously supplied G-3-P was found in the LPA plus PA, DAG, and TAG pools, as one would expect under conditions in which the Kennedy pathway was operating. About 25% of the \(^{3}H\) in acyl lipids was distributed in the TAG fraction (Table VI, experiment A). Very small amounts (1 to 5%) of the \(^{3}H\) from G-3-P were detected in the PC, LPC, and PE pools. Only the TAG pool became labeled with 14C from erucoyl-CoA. This is consistent with the fact that 14C-22:1-CoA was incorporated only via DGAT. Furthermore, the large percentage of \(^{3}H\) found in the LPA, PA, and DAG pools (approximately 75%) suggests that endogenous acyl-CoA species available for acylating the sn-1 and -2 positions were not limiting. When analyzed by radio-HPLC for both \(^{3}H\) and 14C simultaneously, the double-labeled TAG fraction showed the presence of both isotopes in the four monoerucyl TAG species (Table VII). The rate of \(^{3}H\) incorporation into all TAGs remained constant at about 14 to 15 pmol min\(^{-1}\) mg protein\(^{-1}\), while the rate of 14C incorporation varied in ratios consistent with the pattern shown in Figure 5. While not unequivocal, these findings strongly suggested that the \(^{3}H\) glycerol backbone originating in G-3-P was incorporated uniformly into all DAG species, via LPA and PA. Subsequently, 14C-erucoyl moieties were incorporated onto DAGs. Thus, the ratio of 14C/\(^{3}H\) in each TAG species reflected the relative proportions of the individual endogenous DAG species available to accept 14C-erucoyl moieties.

To test whether G-3-P was capable of regulating the conversion of PC to DAG during TAG biosynthesis in vitro in the MD embryo system, a double label experiment was performed, examining the ability of 200 \( \mu \text{M} (\text{[^3]H})\text{PC} \) to act as an acyl acceptor for 14C-22:1-CoA, both in the absence and presence of unlabeled 500 \( \mu \text{M} \text{G-3-P} \) (Table VI, experiments B and C, respectively). These data were then compared with that obtained when 500 \( \mu \text{M} (\text{[^3]H})\text{G-3-P} \) was tested alone as an acceptor for 14C-22:1-CoA (Table VI, experiment A).

### Table VI. Distribution of Radiolabel in Acyl Lipids Formed by a Homogenate from Late Cotyledonary MD Embryos of B. napus cv Reston in Double Label Experiments

<table>
<thead>
<tr>
<th>Experiment Reaction Conditions</th>
<th>Distribution of (^{3}H) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPA + PA PC LPC DAG TAG PE</td>
</tr>
<tr>
<td>A/(^{3}H)G-3-P</td>
<td>37 1 5 31 25 1</td>
</tr>
<tr>
<td>B/(^{3}H)PC</td>
<td>16 53 11 8 9 3</td>
</tr>
<tr>
<td>C/(^{3}H)PC + G-3-P</td>
<td>14 41 10 9 25 1</td>
</tr>
</tbody>
</table>

\(^{a}\) \(^{14}C\)-22:1-CoA was the acyl donor in all cases. \(^{b}\) \(^{14}C\)-22:1 was incorporated essentially only into the TAG fraction and was esterified at the sn-3 position.

### Table VII. Incorporation of Radiolabel into TAG Species Formed by a Homogenate from Mid-Late Cotyledonary MD Embryos of B. napus cv Reston Incubated in the Presence of \([^{14}C]22:1\text{-CoA and 500 \( \mu \text{M} \text{G-3-P} \)]) (Table VI, experiment A).

<table>
<thead>
<tr>
<th>TAG Species</th>
<th>Incorporation of Radiolabel</th>
<th>Ratio (^{14}C/^{3}H)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total pmol (pmol min(^{-1}) mg protein(^{-1}))</td>
<td>14C</td>
</tr>
<tr>
<td>18:3/18:1/22:1</td>
<td>350 (14.6)</td>
<td>280 (11.7)</td>
</tr>
<tr>
<td>18:2/18:1/22:1</td>
<td>310 (13.0)</td>
<td>640 (26.7)</td>
</tr>
<tr>
<td>18:1/18:1/22:1</td>
<td>340 (14.2)</td>
<td>1190 (49.7)</td>
</tr>
<tr>
<td>16:0/18:1/22:1</td>
<td>370 (15.5)</td>
<td>70 (2.9)</td>
</tr>
</tbody>
</table>

\(^{a}\) Does not imply stereospecificity of acyl moieties at the sn-1 and -2 positions. \(^{b}\) \(^{14}C\)-22:1 was incorporated exclusively into the TAG fraction at the sn-3 position. \(^{c}\) These ratios reflect the relative proportions of \(^{14}C\)-labeled TAG species depicted in Figure 5.
[14]H]PC alone (Table VI, experiment B) was not a very effective 14C-erucyl acceptor. When compared with [14]H]G-3-P (Table VI, experiment A), there was a threefold decrease in the proportion of H allocated to TAGs. However, when [14]H]PC was supplemented with unlabeled G-3-P (Table VI, experiment C), the proportion of H in TAGs increased dramatically, to a level equivalent to that observed with [14]H]G-3-P alone (Table VI, experiment A). H in PC decreased in concert with the increase of H in TAGs in Table VI, experiment C. Furthermore, the proportion of H in DAG remained constant in Table VI, experiments B and C, suggesting that DAG was a true intermediate in the conversion of PC to TAG, with label accumulating in the latter. The presence of H in the LPC fraction was most likely due to the reverse action of an acyl-CoA:lysophosphatidylcholine acyltransferase, converting [14]H]PC to [14]H]LPC plus acyl-CoA in the presence of CoASH, which was present in our reaction mixtures. The presence of this enzyme has been documented in developing oilseeds (44).

In Table VI, experiment C, the unlabeled glycerol backbone would be expected to move from G-3-P to LPA, PA, and then DAG via the typical Kennedy Pathway, as in Table VI, experiment A. The DAG pool would then be acylated with 14C-erucyl moieties to give 14C]TAGs. In addition, the DAG pool could equilibrate with the [14]H]PC pool, first via forward and then reverse reactions of CPTase (41, 42). Thus, a proportion of the H originating in the PC pool would be expected to enter the DAG pool, and subsequently, the TAG pool. The result would be TAGs containing both H and 14C. However, if the increased incorporation of H from PC into TAGs observed in Table VI, experiment C was being driven solely by the mass effect of adding unlabeled G-3-P, one would expect a dilution of the proportion of H in DAGs and TAGs, as compared with the control (Table VI, experiment B). This did not occur. Furthermore, a comparison of the rates of H and 14C incorporation into the TAG species 18:1/18:1/22:1, in experiments A, B and C (Table VIII), confirmed that in the presence of unlabeled G-3-P, the rate of incorporation of both H from PC (primarily dioleoyl PC), and 14C from 22:1-CoA, into 14C/18:1/18:1/22:1 TAG, was essentially restored to that observed in the presence of H G-3-P alone.

Because the rate of incorporation of H from [14]H]PC into [14]H]TAGs was not significantly diluted by the presence of a relative excess of unlabeled backbone supplied as G-3-P (Table VIII), the current findings are consistent with a direct effect of G-3-P on CPTase activity, stimulating the conversion of [14]H]PC to [14]H]DAG, and ultimately, [14]H]TAG. While we are unaware of any previous direct tests of the effect of G-3-P on the transfer of radiolabeled PC moieties to DAG and TAG pools in oilseeds, the present results are generally consistent with trends reported earlier in safflower microsome experiments. In one study (42), a G-3-P “chase” was shown to enhance the transfer of 14C originating in [glycerol-14C]LPC to [14]C]TAGs, via 14C]PCP, whereas proportions of radiolabel in PAs and DAGs remained relatively constant. It has also been shown that the transfer of radiolabel from a PC fraction to DAGs was enhanced by supplying unlabeled DAGs in the mesosomal preparations from safflower cotyledons (41).

It should be noted that a proportion of the H supplied in PC ended up in the LPA plus PA pool (Table VI, experiments B and C). This was presumably due to the activity of phospholipase D in the homogenate. This enzyme is known to be present in developing seeds (16, 39). However, the proportion of H in LPA plus PA (approximately 15%) resulting from the hydrolysis of [14]H]PC, remained constant in the presence (Table VI, experiment C) or absence (Table VI, experiment B) of G-3-P. Proportions of H in LPA plus PA were not significantly diluted by the relative excess of unlabeled G-3-P, yet H in PC decreased, whereas that in TAGs increased. These trends add further credence to the argument that the main effect of G-3-P in the presence of [14]H]PC was to directly stimulate transfer of acylated glycerol backbones from PC to DAG pools via CPTase, followed by conversion of DAGs into TAGs via DGAT.

If Kennedy Pathway intermediates can also affect this interconversion in a manner similar to G-3-P, this could explain the similar 14C TAG patterns observed regardless of the acyl "acceptor" tested. The DAGs, released from an endogenous PC pool, would reflect the acyl composition of the latter, regardless of the exogenous acceptor supplied. These DAGs would then be expected to incorporate 14C-22:1 moieties into the sn-3 position via the DGAT reaction, always yielding the same radiolabeled TAG pattern. Clearly, an exception to this general trend would arise only in a situation in which an exogenous DAG substrate such as the unnatural sn-1,2-dierucin was supplied. In this case, in addition to the TAGs arising from the endogenous DAG acceptor pool, one would also expect to see some radiolabeled trierucin produced, as was observed (Fig. 4).

**Test of Postsynthetic Modification of Preformed TAGs**

Since erucyl moieties are found endogenously in the sn-1 position of TAGs from both B. napus MD embryos (this

**Table VIII. Incorporation of Radiolabel into TAG, 18:1/18:1/22:1, Formed by a Homogenate from Late Cotyledonary MD Embryos of B. napus cv Reston in Double Label Experiments**

A homogenate was prepared from developing MD embryos of B. napus cv Reston at a mid-late cotyledonary stage and incubated with [1-14C]22:1-CoA in the presence of 500 μM L-(2-3H(N))G-3-P (experiment A), 200 μM L-(2-3H(N))PC (experiment B), or 200 μM L-(2-3H(N))PC + 500 μM G-3-P (experiment C). After 30 min, radioactivity in 18:1/18:1/22:1 was determined by radio-HPLC.

<table>
<thead>
<tr>
<th>Experiment/Reaction Conditions</th>
<th>Incorporation of Radiolabel into 18:1/18:1/22:1</th>
<th>Ratio of 14C/14H</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/ + 3H-G-3-P</td>
<td>340 (14.2)</td>
<td>3.5</td>
</tr>
<tr>
<td>B/ + 3H-PC</td>
<td>20 (0.8)</td>
<td>7.5</td>
</tr>
<tr>
<td>C/ + 3H-PC + G-3-P</td>
<td>315 (13.2)</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* 14C-22:1-CoA was the acyl donor supplied in all cases. 22:1 was incorporated into the TAG fraction only at the sn-3 position.
lipids possessing similar characteristics to those found in developing zygotic embryos of the same cv. In vitro, in the presence of G-3-P, homogenates prepared from the MD embryos are capable of TAG bioassembly, incorporating \(^{14}\text{C}-18:1\) moieties into the sn-1 and -2 positions, while \(^{14}\text{C}-22:1\) moieties from erucoyl-CoA are rapidly and specifically inserted into the sn-3 position of TAGs via a highly active erucoyl-CoA:DGAT. DGAT activity was localized primarily in a microsomal fraction. While attempts have failed to isolate from Brassica species, microsomal fractions capable of significant incorporation of radiolabeled erucoyl moieties into TAGs in the presence of G-3-P (4, 23, 43, 44), a microsomal preparation from B. napus MD embryos is highly active in this regard. G-3-P and a number of acyl lipids appear to enhance erucoyl-CoA:DGAT activity. Preliminary evidence suggests that G-3-P may facilitate the release of DAG acceptors from a PC pool via a direct stimulation of CPTase activity. It is also possible that G-3-P is acting as an allosteric effector of DGAT. However, this hypothesis can only be tested upon purification of the enzyme. The microsome-derived embryo system is an attractive alternative to zygotic embryos as an enzyme source for studies of lipid biosynthesis enzymes. It is easy to obtain specific developmental stages that exhibit high capacities for lipid biosynthesis in vitro.

CONCLUSIONS

In summary, the present study has demonstrated that developing MD embryos of B. napus cv Reston accumulate acyl moieties. This TAG was then incubated with a homogenate prepared from late cotyledonary stage MD embryos of B. napus cv Reston, and \(^{14}\text{C}-22:1\)-CoA was supplied. Using reverse-phase HPLC, the radiolabeled TAG sample was split with 10% going to a mass detector (evaporative analyzer; top panel) and the remaining 90% going to the radioactive flow detector (bottom panel). TAG species were identified by co-chromatography with external TAG standards, located using the mass detector. Abbreviations: LOE, LnOE, etc.: TAG species composed of erucoyl (E), eicosoyl (Ei), linoleoyl (L), linolenyl (Ln), oleoyl (O), and palmitoyl (P) moieties.

Figure 9. Test of postsynthetic modification of preformed TAGs as a mechanism for insertion of erucoyl moieties onto the sn-1 position. sn-1,2 Oleoyl, sn-3 erucylglycerol (OGE) was synthesized as described in "Materials and Methods." This TAG was then incubated with a homogenate prepared from late cotyledonary stage MD embryos of B. napus cv Reston, and \(^{14}\text{C}-22:1\)-CoA was supplied. Using reverse-phase HPLC, the radiolabeled TAG sample was split with 10% going to a mass detector (evaporative analyzer; top panel) and the remaining 90% going to the radioactive flow detector (bottom panel). TAG species were identified by co-chromatography with external TAG standards, located using the mass detector. Abbreviations: LOE, LnOE, etc.: TAG species composed of erucoyl (E), eicosoyl (Ei), linoleoyl (L), linolenyl (Ln), oleoyl (O), and palmitoyl (P) moieties.

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LITERATURE CITED


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