In vitro Hydrolysis of Natural and Synthetic γ-Linolenic Acid-Containing Triacylglycerols by Pancreatic Lipase

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ABSTRACT: The present study compared the *in vitro* hydrolysis of two 18:3n-6-rich oils—evening primrose oil (EPO) and borage oil (BO)—and different synthetic 18:3n-6-containing triacylglycerols (TG). Incubation of EPO and BO with pancreatic lipase lipolyzed 18:3n-6 from the TG species. The rate of lipolysis of TG species containing two or three molecules of 18:3n-6, which comprised 36% of total 18:3n-6 in BO and only 7% in EPO, was significantly slower than those containing only one molecule of 18:3n-6. This was found especially in those with two molecules of linoleic acid, which constituted 20% of total 18:3n-6 in BO, whereas over 80% were present in EPO. In a separate study, various synthetic 18:3n-6-containing TG were also subjected to *in vitro* hydrolysis by pancreatic lipase. Results showed that release of 18:3n-6 from the *sn*-1/*sn*-3 positions was significantly slower when two other stereospecific positions in the same TG molecule were occupied by either palmitic acid (16:0) or monounsaturated (18:1 and 20:1) fatty acids than when occupied by 18:2n-6. The rate of hydrolysis of *sn*-2-γ-linolenyl-*sn*-1(3)-diacylglycerol to form *sn*-2-mono-γ-linolenyl glycerol was also significantly slower when both the *sn*-1 and *sn*-3 positions in TG molecules were occupied by either saturated fatty acids (16:0 and 18:0) or long-chain monounsaturated fatty acids than when occupied by 18:2n-6. These findings suggest that the stereospecific position of 18:3n-6 in TG molecules and the constituent of its neighboring fatty acids modulated availability of 18:3n-6 from 18:3n-6-containing TG or 18:3n-6-rich oils.

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Clinical evidence has shown that diseases associated with impaired Δ^6-desaturase activity may be alleviated by dietary supplementation of γ-linolenic acid (18:3n-6), an immediate Δ^6-desaturation product of linoleic acid (18:2n-6) (1–5). Normally, after its formation, 18:3n-6 is rapidly elongated to 20:3n-6 and, subsequently, Δ^5-desaturated to 20:4n-6 (6). Thus, the occurrence of 18:3n-6 and 20:3n-6 in human and in animal tissues is rare. However, significant amounts of 18:3n-6, mainly in the triacylglycerol (TG) form, may be found in evening primrose oil (EPO, *Oenothera biennis*), borage oil (BO, *Borago officinalis*), and black currant oil *(Ribes nigrum)* (7). Among them, EPO and BO are the most frequently used oils for studying the nutritional and clinical beneficial effects of 18:3n-6.

The content of 18:3n-6 in BO (24–25%) is approximately 2–3-fold that in EPO (7–14%) (7–9). However, results from a mesenteric perfusion study showed that release of prostaglandin E_1 (PGE_1)—a beneficial metabolite of 20:3n-6—was significantly greater in EPO-supplemented than in BO-supplemented rats (10). This finding suggests that formation of 20:3n-6 and its metabolite, PGE_1, does not correlate to content of 18:3n-6 in BO or EPO, and that bioavailability of 18:3n-6 is not equal between EPO and BO. On the other hand, results from a separate study have shown no significant difference in tissue 18:3n-6 and 20:3n-6 levels between animal groups given equal amounts of dietary 18:3n-6 either as BO or EPO (11).

Comparing the fatty acid compositions in oils and in the chylomicronal TG (in plasma or in thoracic duct) after absorption may provide a simple indication to what extent the 18:3n-6 in the supplemented oil is digested and absorbed. Because the distribution patterns of 18:3n-6 in 18:3n-6-containing TG differ among different oils, analysis of tissue fatty acids cannot adequately explain how and to what extent the stereospecific position of 18:3n-6 and the copresence of other fatty acids in the TG molecules affects the absorption of 18:3n-6 (12–16). Moreover, pancreatic lipase hydrolyzes TG of different molecular weights at different rates (17).

For this report, we examined the *in vitro* hydrolysis of natural and synthetic 18:3n-6-rich TG by pancreatic lipase. In the first experiment, we compared distributions and rates of *in vitro* hydrolysis of different 18:3n-6-containing TG molecular species in two natural 18:3n-6-rich oils, BO and EPO. In the second experiment, we examined hydrolysis rates of different synthetic 18:3n-6-containing TG. The aim was to distinguish whether different stereospecific positions of γ-linolenic acid in the TG molecule and its association with other fatty acids in the same TG molecule affected the release of 18:3n-6 from TG molecules.

EXPERIMENTAL PROCEDURES

Chemicals. Porcine pancreatic lipase (grade III), DL-1,2-isopropylidene glycerol, 4-dimethylaminopyridine (4-DMAP),

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dicyclohexylcarbodiimide (DCC), and boric acid were obtained from Sigma Chemical Company (St. Louis, MO). 1,3-Benzylidene glycerol was purchased from Serdary Research Laboratories, Inc. (Toronto, Ontario, Canada). Sodium borohydride and 1,3-dihydroxyacetone were from Aldrich Chemical Co., Inc. (Milwaukee, WI). BF_3–methanol was supplied by Pierce Chemical Co. (Rockford, IL). Isopropanol, chloroform, acetonitrile, and other organic solvents of high-performance liquid chromatography (HPLC) grade were supplied by British Drug Houses (Toronto, Ontario, Canada). The TG standards, such as tri-γ-linolenin (GGG), linoleoyl-di-γ-linolenin (LGG), di-linoleoyl-γ-linolenin (LLG), and trilinolein (LLL), were obtained from Nu-Chek-Prep (Elysian, MN) or synthesized from chromatographically pure 18:2n-6 and 18:3n-6. The TG fractions in the cold-pressed olive oil, EPO (Efamol Ltd., Guildford, United Kingdom), and BO (Callanish Ltd., Scotland, United Kingdom) were purified by silicic acid column chromatography.

HPLC fractionation of TGs. HPLC analysis was carried out with a Beckman System Gold programmable solvent module 126 (Beckman Instruments, Palo Alto, CA). Two identical reverse-phase columns, Supelcosil LC-18 (5 μm, 250 mm × 4.6 mm i.d.; Supelco, Bellefonte, PA), connected in series and housed in a programmable column oven (30°C), were used for separation and analysis of the highly unsaturated TG molecular species present in EPO and BO. A solvent mixture of isopropanol/acetonitrile (35:65, vol/vol) was used to isocratically elute the TG fractions in the natural oils (18). A computer was used to automatically offset the absorbance of the mobile phase and justify the actual readings of the eluents. For preparative analysis of oils (200 mg/mL in chloroform), separation of TG components was detected with an ultraviolet detector (Model 166; Beckman) at 210 nm. The eluted TG fractions were collected manually for fatty acid analysis. Three or more collections were pooled to ensure that the quantity of each eluted TG fraction collected was sufficient. To assess the relative distribution of all TG fractions present in oils, a mass (light-scattering) detector (ACS model 740/14; Applied Chromatography System Ltd., Macclesfield, England) was used. The settings on the mass detector have been described previously (19), and were used as follows: attenuation range, 16; photomultiplier sensitivity, 5; time constant, 5 s; evaporator set, 40; internal air pressure, 27 psi. The output signal integration and data analysis were performed by an IBM PS/2 computer (model 50) with Gold Software (version 3.1). In addition to mass detection, we have also calibrated the percentage weight distributions of TG molecular species in both EPO and BO with triheptadecanoin (tri-17:0) as an internal standard, analyzed by gas–liquid chromatography (GLC). The results obtained from both methods were comparable (data not shown).

GLC analysis of fatty acids in oil TGs. The collected TG fractions were evaporated to dryness under nitrogen and redissolved in 1 mL chloroform. To convert TG into fatty acid methyl esters (FAME), an aliquot (200 μL) of TG solution, a known amount of internal standard (triheptadecanoin dissolved in chloroform), and an equal volume of benzene were pipetted into a test tube, flushed with a stream of nitrogen, screw-capped, and heated for 1 h at 90°C with 2 mL 14% BF_3–methanol (20). The FAME mixture was then analyzed with a fused-silica capillary column (Supelcomega, 50 m × 0.25 mm i.d.; Supelco) in a Hewlett-Packard Model 5890 gas–liquid chromatograph equipped with a flame-ionization detector, as described previously (21).

Synthesis of sn-*1(3)-mono-[1-^{14}C]-γ-linolenoyl*-sn-*2,3(1)-diradylglycerols* (**GXX**). The procedure described by Jensen and Pitas (22) was used to synthesize **GXX**, where **G** represents the radiolabelled γ-linolenic acid, and X represents other nonradiolabelled fatty acids, such as palmitic (P), stearic (S), oleic (O), linoleic (L), or erucic acid (E). Briefly, the starting material, (±)1,2-O-isopropylidene-*rac*-glycerol (solketal), was esterified with [1-^{14}C]-γ-linolenic acid in the presence of one equivalent amount of 4-DMAP and DCC in carbon tetrachloride. The synthesized *sn*-1(3)-mono-[1-^{14}C]-γ-linolenoyl glycerol isopropylidene ester was then purified by dry column–flash chromatography (DCFC; 32–74 μm silica gel), and later hydrolyzed by refluxing with an excess of boric acid in 2-methoxyethanol to form *sn*-1(3)-monoacylglycerol.

Purified *sn*-1(3)-mono-[1-^{14}C]-γ-linolenyl glycerol was then esterified with two equivalents of either P, S, O, L, or E in the presence of two equivalents of 4-DMAP and DCC, respectively (23). The resulting mixture of *sn*-1-mono-[1-^{14}C]-γ-linolenyl-*sn*-2,3- and *sn*-3-mono-[1-^{14}C]-γ-linolenyl-*sn*-1,2-diradylglycerols was purified by DCFC as in the previous paragraph. The final yield was 50–60%.

Synthesis of sn-*2-mono-[1-^{14}C]-γ-linolenyl*-sn-*1,3-diradylglycerols* (**XGX**) *or* sn-*2-mono-[1-^{14}C]-linoleoyl*-sn-*1,3-diradylglycerols* (**XLX**). The syntheses of **XGX** and **XLX** followed the method described by Serdarevich and Carroll (24). The starting material for this procedure was 1,3-benzylidene glycerol. This compound was esterified with one equivalent amount of either [1-^{14}C]-γ-linolenic acid or [1-^{14}C]-linoleic acid in the presence of 4-DMAP and DCC to yield approximately 50% of *sn*-2-mono-γ-linolenyl- or *sn*-2-mono-linoleoyl-1,3-benzylidene glycerol ester. The benzylidene moiety was cleaved by reflux with an excess of boric acid in methoxyethanol. The *sn*-2-mono-acylglycerol was extracted with diethyl ether, concentrated, and purified by column chromatography on 10% boric acid-impregnated silica gel. Boric acid was required to prevent migration of fatty acid from the *sn*-2 to *sn*-1 position.

Purified *sn*-2-mono-[1-^{14}C]-γ-linolenyl or *sn*-2-mono-[1-^{14}C]-γ-linoleoyl-glycerol was esterified with two equivalents of P, S, O, L, or E in the presence of two equivalents of 4-DMAP and DCC, respectively (23). The synthesized *sn*-2-mono-[1-^{14}C]-γ-linolenyl- or *sn*-2-mono-[1-^{14}C]-linoleoyl-*sn*-1,3-diradylglycerols were purified by DCFC as described previously. The final yield was in the region of 30%.

Synthesis of sn-*1,3-di-[1-^{14}C]-γ-linolenyl*-sn-*2-mono-radylglycerol* (**GXG**) *or* sn-*1,3-di[1-^{14}C]-linoleoyl*-sn-*2-mono-radylglycerol* (**LXL**). The method described by Bentley and McCrae (25) was used for the synthesis of **GXG** and

LXL. The starting material for synthesis of **GXG** and **LXL** was dihydroxyacetone. One mole of dihydroxyacetone was esterified with 2 moles of either [1-^{14}C]-linoleic or [1-^{14}C]-γ-linolenic acid in the presence of 2 moles of 4-DMAP and DCC, respectively, to form 1,2,3-trihydroxypropane-1,3-di-[1-^{14}C]-linoleate or 1,2,3-trihydroxypropane-1,3-di-[1-^{14}C]-γ-linolenate, which was purified by DCFC. The purified product was dissolved in chilled (5°C) tetrahydrofuran (1.5 mL) and water (100 μL), and treated portionwise with neutral sodium borohydride. After reduction, the solution was diluted with chloroform, washed with water, aqueous $NaHCO_3$ and water, and was dried over magnesium sulfate. After evaporation, 1,2,3-trihydroxypropane-1,3-di-[1-^{14}C]-linoleate or 1,2,3-trihydroxypropane-1,3-di-[1-^{14}C]-γ-linolenate, shown as a colorless oil, was esterified with one equivalent amount of either O, L, or G in the presence of one equivalent amount of 4-DMAP and DCC, respectively (22). The resulting TG, *sn*-1,3-di-[1-^{14}C]-γ-linolenyl-*sn*-2-monoradylglycerol (**GXG**) or *sn*-1,3-di-[1-^{14}C]-linoleoyl-*sn*-2-monoradylglycerol (**LXL**) was purified as described previously. The overall yield was approximately 60–70%.

Confirmation of the purity of synthetic TGs. The purity of synthetic TGs was confirmed by (i) thin-layer chromatography (TLC) to examine if any trace of free fatty acids (FFA), monoacylglycerols (MG), or diacylglycerols (DG) existed; (ii) GLC to examine whether the constituent fatty acids in the synthetic TG were in a correct ratio; (iii) HPLC to determine if any undesired TG molecular species were presented (18); and (iv) random hydrolysis with Grignard reagent to determine whether the specific fatty acids were esterified to the desired positions (26,27).

Digestion of natural and synthetic TGs with pancreatic lipase. Both natural and synthetic TGs were subjected to hydrolysis by pancreatic lipase by following a method modified from those described previously by Luddy *et al.* (28) and Yang *et al.* (29). Briefly, approximately 5 mg of TG were dissolved in 100 μL hexane and transferred to a test tube. After evaporation to dryness, 0.5 mL of 1 M tris[hydroxymethyl]-aminomethane buffer (pH 8.0), 0.2 mL 22% $CaCl_2$ solution, and 0.5 mL of 0.1% bile salt solution were added. The mixture was allowed to equilibrate at 40°C for 1 min in a water bath. The incubation began when 500 μL of pancreatic lipase preparation (2 mg/mL in Tris buffer, pH 8.0) was added to the mixture. All analyses were performed in triplicate in screw-cap vials in a shaking bath (G24 Environment Incubator Shaker; New Brunswick Scientific, Edison, NJ) at 37°C for variable intervals (0, 5, 10, and 20 min for the natural TG and 0, 1, 2, 3, 4, and 5 min for the synthetic TG). The reaction was stopped by adding 1 mL ethanol, followed by 1 mL diluted HCl (6 M). The lipids were extracted into diethyl ether (3 × 4 mL), washed twice with 4 mL distilled water, dried over anhydrous sodium sulfate, and concentrated with a nitrogen stream. The lipid classes TG, DG, MG, and FFA were separated by TLC on silica gel G plates developed with the solvent system hexane/diethyl ether/formic acid (80:20:2, vol/vol/vol). After methylation with 14% BF_3–methanol (20), the methyl esters of fatty acids from different lipid fractions were analyzed by GLC, as described previously (21). To quantitate the relative amount of different lipid fractions after incubation, a known amount of heptadecanoic acid as an internal standard was added to TG, DG, MG, and FFA prior to methylation. In a separate study, the weight distribution of different TG molecular species in the residual TG fraction after hydrolysis by pancreatic lipase was examined by HPLC. In studying the hydrolysis of the radiolabelled synthetic TG, the percent distribution of radioactivity in each lipid class was determined by a TLC scanner (Dunnschicht Scanner II; Laboratorium Professor Dr. Berthold, Wildbad, Germany).

Statistics. Data are presented as mean ± SD of three analyses. The significance of differences between means was assessed with analysis of variance by computer.

RESULTS AND DISCUSSION

Separation of oil TGs. Results in the preliminary study indicated that the described HPLC method separated the mixture of TG (GGG, LGG, LLG, and LLL) baseline-to-baseline, although the positional isomers (e.g., GGL, GLG, and LGG) could not be differentiated. Results also showed that a positive log relation existed between the retention time of TG molecular species and their theoretical carbon number (TCN), defined as $TCN = ECN - \Sigma U_i$, as reported previously by El-Hamdy and Perkins (30). In the formula, ECN (equivalent carbon number) represents the actual number of carbon in fatty acids less twice the number of double bonds, and U_i represents a correcting factor for specific fatty acids (21). El-Hamdy and Perkins (30) have reported the U_i value to be zero for saturated fatty acids, 0.6 for oleic acid, and 0.8 for linoleic acid. In the present study, by isocratically co-eluting a mixture of TG standards (GGG, LGG, LLG, and LLL) and TG purified from olive oil (rich in OLL, OOL, POL, PPL, OOO, and SOO), we were able to assess the U_i value for γ-linolenic acid to be 0.4. Using the positive log relation between TCN and retention times, and a computer program based on fatty acid composition and random distribution, we have identified and calculated the weight distribution of possible TG molecular species in EPO and BO.

Figure 1 and Table 1 show that EPO contained 13 detectable TG fractions, and BO contained 25 TG fractions. Due to high degree of unsaturation, the retention times of 18:3n-6-containing TG molecules were shorter than other TG species. However, a significant proportion of 18:3n-6-containing TG species in BO were associated with saturated and monounsaturated n-9 fatty acids. This association slowed down the elution, and, hence, a longer elution time was needed to elute all TG fractions in BO.

Hydrolysis of TGs in EPO and BO by pancreatic lipase. After purification by column chromatography, the TG fractions from EPO and BO were subjected to *in vitro* hydrolysis by pancreatic lipase for various time intervals (0, 5, 10, and 20 min). The rates of hydrolysis of TG and formation of DG and MG were comparable between BO and EPO. However,

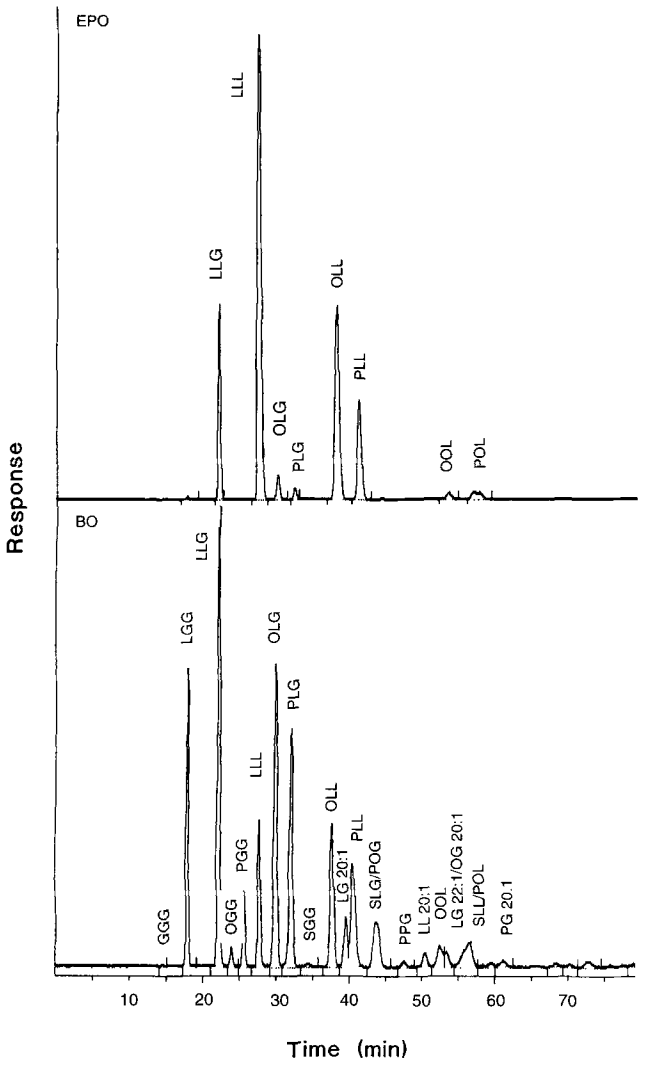

FIG. 1. High-performance liquid chromatography separation of triacylglycerols of evening primrose oil (EPO) and borage oil (BO) on double Supelcosil LC-18 columns (5 μm, 4.6 mm i.d. × 250 mm; Supelco, Bellefonte, PA). Column temperature, 30°C; mobile phase, isopropanol/acetonitrile (35:65, vol/vol) at a flow rate of 2 mL/min. Detection by a mass (light-scattering) detector. L, linoleic acid; G, γ-linoleic acid; O, oleic acid; P, palmitic acid; S, stearic acid.

TABLE 1
Distribution (% weight) of Total Fatty Acids Quantitated by Gas–Liquid Chromatography and Triacylglycerol (TG) Fractions Separated by Reverse-Phase High-Performance Liquid Chromatography from Borage Oil (BO) and Evening Primrose Oil (EPO)[a]

	BO	EPO
Fatty acid		
16:0, P	10.7[b]	5.9
18:0, S	3.0	1.8
18:1n-9, O	15.4	7.5
18:2n-6, L	38.1	74.8
18:3n-6, G	24.8	9.3
20:1n-9, S	4.0	—
22:1n-9, E	2.2	—
TG fraction		
GGG	0.24	—
LGG	9.84	0.71
LLG	15.41	17.63
OGG	0.83	trace
PGG	2.39	trace
LLL	10.11	54.33
OLG	12.30	1.55
PLG	9.53	1.19
SGG	0.25	—
OLL	8.17	13.74
LGD	2.53	—
PLL, OOG	8.93	7.94
SLG, POG	4.97	trace
PPG	1.47	—
LLD	4.11	—
OOL	4.05	0.76
OGD	0.35	—
POL, SLL	0.25	2.15
LGE	0.55	—
PGD	0.78	—
SOG	0.23	—
PPL, POO	0.39	trace
LLE	0.61	—
SOL, PLD, OGE	0.22	—
POO, PSL, PPO	0.86	—

[a]P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; G, γ-linoleic acid; D, 20:1n-9; E, erucic acid.
[b]Each value represents mean of three determinations.

fatty acid compositions in the residual TG and the yield of DG and MG fractions derived from EPO and BO were very different (Fig. 2). With respect to 18:2n-6, the initial level of 18:2n-6 in the TG fraction was higher in EPO than in BO, but the pattern of changes of 18:2n-6 in response to hydrolysis was not different between EPO and BO—they decreased progressively in the residual TG and the DG fractions but were relatively constant in the MG fraction. With respect to 18:3n-6, the levels of 18:3n-6 were higher in BO than in EPO. Hydrolysis increased progressively the levels of 18:3n-6 in the residual TG and the DG fractions, but not in the MG fraction. The increase of 18:3n-6 in the residual TG and in the formed DG fraction was significantly greater in BO than EPO. The levels of 18:1n-9 in the residual TG fractions were similar to those in the initial TG fractions. They were also relatively constant in the DG and MG fractions at different hydrolysis times.

Figure 3 shows the distribution (% weight) of different γ-linolenic acid-containing TG molecular species in the residual TG fractions after EPO and BO were hydrolyzed by pancreatic lipase. The proportions of TG molecular species that contained two or three molecules of 18:3n-6 (GGG, LGG, OGG, and PGG) were initially higher in BO than in EPO. During hydrolysis, the proportions of these TG species were increased more rapidly in the residual TG fraction derived from BO than from EPO. The levels of TG species that contained only one molecule of 18:3n-6, such as LLG, OLG, and PLG, were also higher in BO than in EPO. During hydrolysis, the proportions of LLG in the residual TG fraction increased during the first 10 min but decreased rapidly after 10 min of incubation. The proportions of OLG and PLG in the residual TG fractions from BO and EPO, on the other hand,

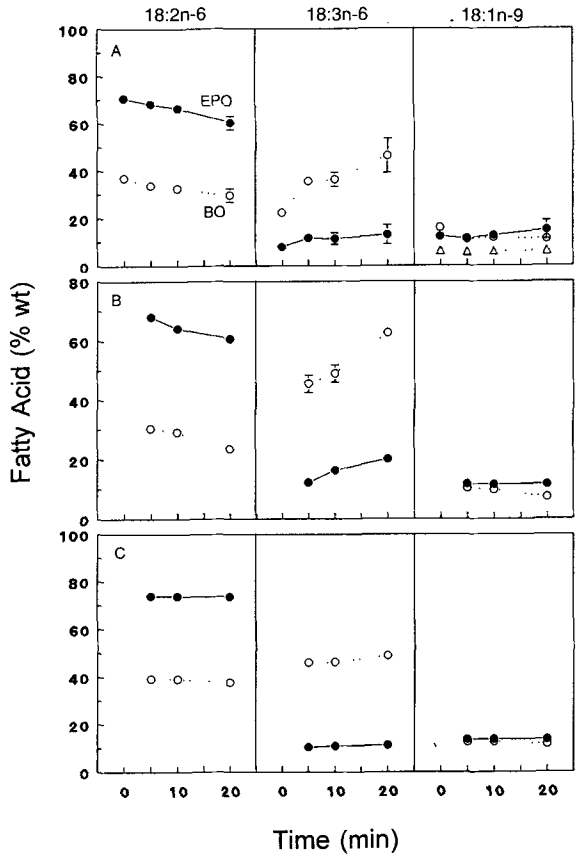

FIG. 2. Distribution (% weight) of 18:2n-6, 18:3n-6, and 18:1n-9 in residual triacylglycerols (panel A), diacylglycerols (panel B), and monoacylglycerols (panel C) in purified triacylglycerol fraction of evening primrose oil (EPO, ●—●) and borage oil (BO, ○···○) subjected to *in vitro* hydrolysis by pancreatic lipase for 5, 10, and 20 min. Each data point represents mean ± SD of three determinations.

responded differently to hydrolysis. The proportions of OLG and PLG in EPO increased progressively with incubation time, whereas those in BO decreased after 5 min of incubation. These results clearly show that TG in BO and EPO were hydrolyzed by pancreatic lipase at different rates.

Hydrolysis of synthetic TGs by pancreatic lipase. Table 2 shows the hydrolysis rates of various synthetic *sn*-1(3)-mono-[1-^{14}C]-γ-linolenoyl-2(1,3)-diradylglycerols in response to the action of pancreatic lipase. The levels of radioactivity in the DG fraction, which reflected the rates of hydrolysis of GXX → GX, were in the following order: GLL > GPP = GEE > GOO = GSS.

The levels of radioactivity in the FFA fraction, which derived from two possible routes of hydrolysis:

$$\text{GXX} \rightarrow \text{free G} + \text{XX} \quad [1]$$

$$\text{GXX} \rightarrow [\text{free X} + \text{GX}] \rightarrow \text{free G}, \quad [2]$$

were in the order: GSS > GLL > GPP = GEE > GOO. Overall, GLL (32.6%), among all GXX, has the highest hydrolysis rate. To assess the relative rates of the two hydrolysis routes, the ratios of radioactivity distributed in the DG and

TABLE 2
Hydrolysis of Synthetic [1-^{14}C]-γ-Linolenate-Containing Triacylglycerols by Pancreatic Lipase: Distribution of Radioactivity in Hydrolysis Products[a]

	% Radioactivity			Ratio	
Substrate	DG	FFA	MG	DG/FFA	DG/MG
GPP	12.0 ± 0.3[a]	4.7 ± 0.5[a]	—	2.6	—
GSS	8.7 ± 1.9[b]	10.1 ± 0.2[b]	—	0.9	—
GOO	9.7 ± 7.9[a,b]	1.3 ± 1.4[c]	—	7.5	—
GEE	12.0 ± 1.5[a]	3.8 ± 0.8[a]	—	3.2	—
GLL	23.9 ± 4.5[c]	8.7 ± 2.3[b]	—	2.7	—
PGP	6.7 ± 1.5[a]	—	4.0 ± 2.0[a]	—	1.7
SGS	5.9 ± 0.1[a]	—	4.1 ± 1.9[a]	—	1.4
OGO	12.6 ± 0.5[b]	—	8.4 ± 1.1[b]	—	1.5
EGE	10.3 ± 1.8[b]	—	4.7 ± 0.2[a]	—	2.2
LGL	12.8 ± 4.1[b]	—	9.3 ± 1.4[b]	—	1.4

[a]Abbreviations: DG, diacylglycerols; FFA, free fatty acids; MG, monoacylglycerols; E, 20:1n-9; G, 18:3n-6; L, 18:2n-6; O, 18:1n-9; P, 16:0; S, 18:0; **G**, radiolabelled γ-linoleic. All substrates were provided in equimolar quantity, and reaction time was 3 min. Values with different superscripts are significantly different from each other at $P < 0.05$.

FFA fractions (DG/FFA) were calculated. With exception of GSS, the ratios were greater than 1, suggesting that hydrolysis of GXX favored the route of GXX →[GX + free X] over that of GXX → free G + XX, and the hydrolysis of GX → free G was low. Because no radioactivity was observed in the MG fractions, this indicates that there was no significant migration of G from *sn*-1 or *sn*-3 to *sn*-2 under the present experimental conditions.

In a separate experiment, various XGX, where radiolabeled 18:3n-6 locates at the *sn*-2 position, were subjected to hydrolysis by the action of pancreatic lipase, and the levels of radioactivity associated with either DG or MG fraction were examined. Results in Table 2 show that the rates of initial hydrolysis of different XGX to form DG, XGX → [GX or XG], were in the following order: LGL = OGO ≥ EGE > PGP = SGS. Comparing the levels of radioactivity in the MG fraction, which possibly derived from the metabolic pathway, XGX → [GX and XG] → mono-G, indicated that the overall hydrolysis was in the order: LGL = OGO > EGE = SGS = PGP. This finding also indicates that XGX were more readily hydrolyzed when X represents unsaturated fatty acid, such as LGL and OGO (19 and 21%, respectively) than when X was a saturated fatty acid, for example SGS and PGP (10 and 10.9%).

To compare the rate of primary hydrolysis of XGX to DG in relation to its subsequent hydrolysis (from DG to MG), the ratios of radioactivity distributed in the DG and MG fractions (DG/MG) were also calculated. Results showed that all XGX, particularly EGE, have high DG/MG ratios, suggesting that these TG species were readily hydrolyzed by pancreatic lipase to form DG (a mixture of XG and GX), but the formed DG species were less readily hydrolyzed to form MG, [XG or GX] → mono-G. We observed no radioactivity associated with the FFA fraction, suggesting that the amount of the *sn*-2-γ-linolenoyl-*sn*-1(3)-acylglycerols underwent acyl migration

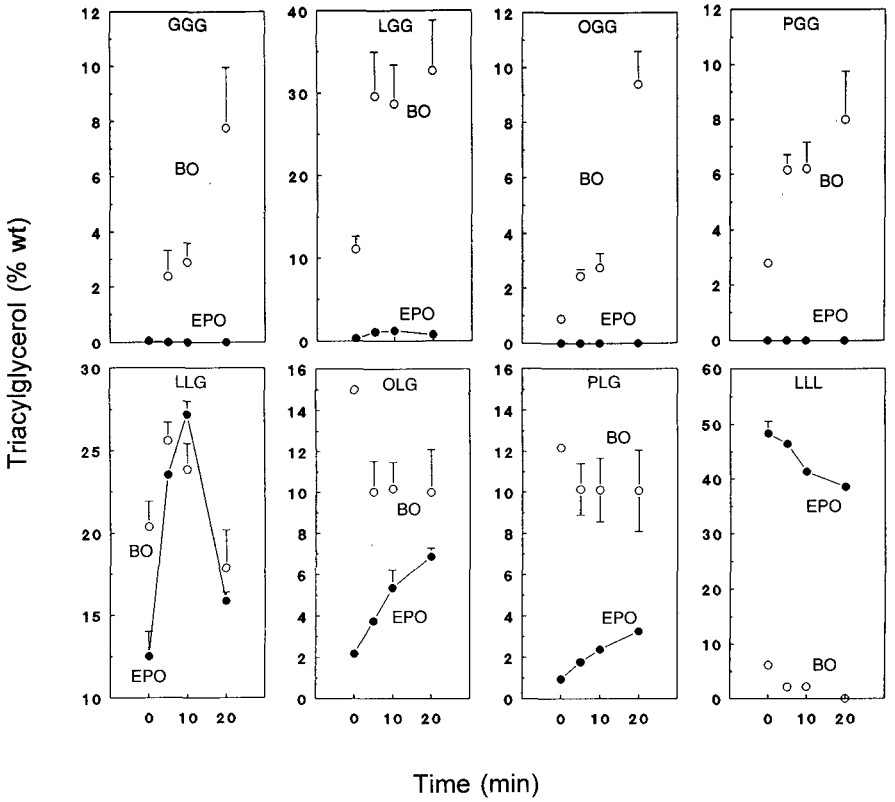

FIG. 3. Distribution (% weight) of residual triacylglycerol molecular species containing three molecules (GGG), two molecules (LGG, OGG, PGG), one molecule (LLG, OLG, PLG), or none of 18:3n-6 (LLL) in evening primrose oil (●—●) and borage oil (○···○) subjected to *in vitro* hydrolysis by pancreatic lipase for 5, 10, and 20 min. Each data point represents mean ± SD of three determinations. Abbreviations as in Figure 1.

to form *sn*-3(1)-γ-linolenoyl-*sn*-1(3)-acylglycerols was insignificant, or these MG species could not be hydrolyzed by pancreatic lipase.

Table 3 compares the rates of hydrolysis of *sn*-1,3-dilinoleoyl- and *sn*-1,3-di-γ-linolenyl-*sn*-2-mono-radylglycerols subjected to the action of pancreatic lipase. Overall, **LXL** were more readily hydrolyzed than were **GXG** by pancreatic lipase. The release of either free **L**, **LX**, or **XL** from **LXL** was greater when the *sn*-2 position was occupied by γ-linolenic than when occupied by linoleic or oleic acid. For **GXG**, the release of free **G**, **GX**, or **XG** was relatively low when the *sn*-2 position in TG molecule was occupied by linoleic acid.

TABLE 3
Comparison of *in vitro* Hydrolysis Rate of [1-^{14}C]-Linoleoyl-Containing and [1-^{14}C]-γ-Linolenoyl-Containing Triacylglycerols by Pancreatic Lipase[a]

Substrate	% Radioactivity		DG/FFA ratio
	DG	FFA	
GOG	3.1 ± 0.6	5.4 ± 0.9	0.6
LOL	6.6 ± 1.2[b]	3.7 ± 0.3[b]	1.8
GLG	0.8 ± 0.4	0.5 ± 0.5	1.6
LLL	10.2 ± 0.5[b]	5.5 ± 0.9[b]	1.9
GGG	3.4 ± 0.9	2.4 ± 0.5	1.5
LGL	13.5 ± 2.5[b]	6.4 ± 1.4[b]	2.2

[a]Abbreviations as in Tables 1 and 2. All substrates were provided in equimolar quantity and reaction time was 3 min.
[b]Significantly different at $P < 0.01$.

When dietary fat is consumed, most of the TG are digested in intestinal lumen by action of pancreatic lipase. Because this enzyme is known to be specific to the primary esters (31,32), TG are hydrolyzed to form FFAs and *sn*-2-monoacylglycerols. The formed FFAs and *sn*-2-monoacylglycerols are then absorbed into intestinal mucosa cells. Within the intestinal cells, they are resynthesized into TG which, assembled with cholesterol, phospholipids, and apolipoproteins into chylomicrons, are then secreted into lymph and pass into plasma *via* thoracic ducts (33). The release of γ-linolenic acid from the fat source as either free acid or as *sn*-2-monoacylglycerol thus modulates its bio-availability.

Results in Table 1 show that over 80% of total 18:3n-6 in EPO, and only about 20% in BO, were found in one single TG molecular species, which contained two molecules of linoleic acid and one molecule of γ-linolenic acid (a mixture of positional isomers, LLG, LGL, and GLL). Although the stereospecific position of 18:3n-6 in different TG positional isomers affected the rate of hydrolysis, the release of *sn*(2)-mono-**G** from **LGL** as compared to other **XGX**, or of free **G** from **GLL** as

compared to other **GXX**, was more rapid (Table 2). Moreover, TG in BO, but not in EPO, contained significant amounts of long-chain (C_{20} + C_{22}) monounsaturated n-9 fatty acids (Table 1). Results in Table 2 show that the release of *sn*-(2)-mono-**G** from the synthetic **EGE** as compared to **LGL**, or free **G** from the synthetic **GEE** as compared to **GLL**, was significantly slower, indicating that hydrolysis of γ-linolenic acid from TG molecules was suppressed by the co-presence of long-chain (C_{20} + C_{22}) monounsaturated fatty acids.

Furthermore, a great proportion of 18:3n-6-containing TG molecular species in BO contain two or three molecules of 18:3n-6 (e.g., GGG, LGG, DGG, PGG, etc.). Results in Figure 3 show that the rate of hydrolysis of these natural 18:3n-6-rich TG was considerably slower. Results in Table 3 also show that the hydrolysis of di-γ-linolenyl-monoacylglycerol (whether in the form of **GGX** or **GXG**) was slower than that of di-linolyl-monoacylglycerol (**LLX** including **LLG**, or **LXL** including **LGL**). This evidence indicates that the availability of γ-linolenic acid from either natural or synthetic TG molecules was limited by the presence of another 18:3n-6 molecule in the same TG molecule.

In summary, results in the present study show that a lower content of LLG, but a higher content of 18:3n-6-containing TG, which also contain long-chain (C_{20} + C_{22}) monounsaturated fatty acids or another 18:3n-6 molecule, might limit the bio-availability of 18:3n-6 in BO in comparison with EPO (10).

REFERENCES

1. Wright, S., and J.L. Burton, *Lancet ii*:1120 (1982).
2. Chaintreuil, J., L. Monnier, C. Colette, P. Crastes de Paulet, A. Orsetti, D. Speilmann, F. Mendy and A. Crastes de Paulet, *Hum. Nutr. Clin. Nutr. 38C*:121 (1984).
3. Pye, J.K., R.E. Mansel and L.E. Hughes, *Lancet ii*:373 (1985).
4. Puolakka, J., L. Makarainen, L. Viinikka and O. Ylikorkala, *J. Reprod. Med. 30*:149 (1985).
5. Horrobin, D.F., *Rev. Contemp. Pharmacothera 1*:1 (1990).
6. Sprecher, H., *Progr. Lipid Res. 20*:13 (1981).
7. Hudson, B.J.F., *J. Am. Oil Chem. Soc. 61*:540 (1984).
8. Ratnayake, W.M.N., D.G. Matthews and R.G. Ackman, *Ibid. 66*:966 (1989).
9. Lawson, L.D., and B.G. Hughes, *Lipids 23*:313 (1988).
10. Jenkins, D.K., J. Mitchell, M.S. Manku and D.F. Horrobin, *Med. Sci. Res. 16*:525 (1988).
11. Raederstoff, D., and U. Moser, *Lipids 27*:1018 (1992).
12. Laakso, P., and W.W. Christie, *Ibid. 25*:349 (1990).
13. Myher, J.J., L. Marai and A. Kuksis, *Ibid. 12*:775 (1977).
14. Kritchevsky, D., *Nutr. Rev. 46*:177 (1988).
15. Small, D.M., *Ann. Rev. Nutr. 11*:413 (1991).
16. Fieler, L.J., Jr., F.H. Mattson and S.J. Fomon, *J. Nutr. 99*:293 (1969).
17. Sampugna, J., J.G. Quinn, R.E. Pitas, D.L. Carpenter and R.G. Jensen, *Lipids 2*:397 (1967).
18. Huang, Y.-S., X. Lin, R.S. Smith, P.R. Redden, D.K. Jenkins and D.F. Horrobin, *Ibid. 27*:711 (1992).
19. Redden, P.R., and Y.-S. Huang, *J. Chromatogr. Biomed. Appl. 567*:21 (1991).
20. Morrison, W.R., and L.M. Smith, *J. Lipid Res. 5*:600 (1964).
21. Huang, Y.-S., R.S. Smith, P.R. Redden, R.C. Cantrill and D.F. Horrobin, *Biochim. Biophys. Acta 1082*:319 (1991).
22. Jensen, R.G., and R.E. Pitas, *Adv. Lipid Res. 14*:213 (1976).
23. Kodali, D.R., D. Atkinson, T.C. Redgrave and D.M. Small, *J. Lipid Res. 28*:403 (1987).
24. Serdarevich, B., and K.K. Carroll, *Ibid. 7*:277 (1966).
25. Bentley, P.H., and W. McCrae, *J. Org. Chem. 35*:2082 (1970).
26. Myher, J.J., and A. Kuksis, *Can. J. Biochem. 57*:117 (1979).
27. Christie, W.W., in *Lipid Analysis,* Pergamon Press, Oxford, England, 1982.
28. Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman and R.W. Riemenschneider, *J. Am. Oil Chem. Soc. 43*:693 (1964).
29. Yang, L.-Y., A. Kuksis and J.J. Myher, *J. Lipid Res. 31*:137 (1990).
30. El-Hamdy, A.H., and E.G. Perkins, *J. Am. Oil Chem. Soc. 58*:867 (1981).
31. Brockerhoff, H., and R.G. Jensen, in *Lipolytic Enzymes,* Academic Press, New York, 1974, pp. 34–89.
32. Leger, C., and M. Charles, in *World Review of Nutrition and Dietetics,* edited by G.H. Bourne, Vol. 35, Karger, Basel, 1980, pp. 96–128.
33. Brindley, D.N., in *Fats in Animal Nutrition,* edited by J. Wiseman, Butterworth's Scientific Ltd., London, 1984, pp. 85–103.

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