Origin of triacylglycerol moiety of plasma very low density lipoproteins in the rat: structural studies

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Abstract We have compared the molecular species composition of the glycerolipids of rat liver and rat plasma very low density lipoproteins (VLDL). There were differences in the stereospecific distribution of the fatty acids in the triacylglycerols (TG) of the liver and of VLDL. While chiral and reversed phase chromatography with mass spectrometry (LC/MS) revealed great similarities in positional distribution and molecular association of the fatty acids between the *sn*-1,2-diacylglycerol (DG) moieties of the VLDL and liver TG, the corresponding *sn*-2,3-DG were distinctly different. The free hepatic *sn*-1,2-DG and the *sn*-1,2-DG moiety contained within hepatic phosphatidic acid showed a maximum 60% homology to the *sn*-1,2-DG contained within the TG of the liver and of VLDL. By contrast, the smaller pool of hepatic free *sn*-2,3-DG was nearly identical to the *sn*-2,3-DG moiety contained in the TG of the liver. These differences between hepatic and VLDL TG indicate that direct transfer of hepatic triacylglycerols is not a major mechanism of VLDL TG formation. On the other hand, the results suggest that stored hepatic TG are largely hydrolyzed to *sn*-1,2-DG and then reesterified to TG before being secreted as VLDL TG. Although an involvement of 2-monoacylglycerol pathway could not be excluded, it probably plays a minor role in VLDL TG formation. Our data suggest that a minimum of 60% of the VLDL TG could have been derived via hydrolysis to DG and reesterification, and a maximum of 40% could have originated via the conventional phosphatidic acid pathway.—**Yang, L-Y., A. Kuksis, J. J. Myher, and G. Steiner.** Origin of triacylglycerol moiety of plasma very low density lipoproteins in the rat: structural studies. *J. Lipid Res.* 1995. **36:** 125–136.

Supplementary key words liver • stereospecific analysis • chromatography • mass spectrometry

Early work by Bar-On et al. (1) and Mayes (2) in rats suggested that the liver contains two triacylglycerol (TG) pools, a large cytoplasmic one that turns over slowly, and a small microsomal one that turns over rapidly. Mooney and Lane (3) estimated that 15% of hepatic TG was secreted without prior hydrolysis. Later, Francone, Kalopissis, and Griffaton (4) and Duerden and Gibbons (5), using cultured hepatocytes, demonstrated that a significant proportion of the TG of very low density lipoprotein (VLDL) is derived by hydrolysis and reesterification of the cytoplasmic TG. Francone et al. (4) suggested that a lysosomal lipase was responsible for the hydrolysis. They suggested this because chloroquine inhibited both lysosomal lipase activity and VLDL secretion. Wiggins and Gibbons (6) estimate that at least 70% of the secreted VLDL-TG can be derived from hydrolysis of stored TG. However, they showed that although chloroquine blocks the activity of lysosomal lipase and VLDL secretion, it does not inhibit intracellular hydrolysis of TG. Therefore they suggested that a different type of lipase was involved. However, the molecular mechanisms and the enzymatic activities responsible for lipolysis and reesterification remain obscure.

Recently, in the intestine, we obtained evidence that also suggested the existence of a lipolysis-reesterification cycle (7). Those studies indicated that free fatty acids, fed as simple alkyl esters, were incorporated into intestinal TG exclusively via the phosphatidic acid (PA) pathway. Detailed examination of the structure of chylomicron TG by chiral phase high performance liquid chromatography (HPLC) suggested that the TG arising via the PA pathway was hydrolyzed to 2-monoacylglycerols (MG) and reesterified prior to incorporation into chylomicrons. The present studies were undertaken in order to establish the sources of the fatty acids in the TG in VLDL. They involved the determination of both the structure of glycerides and the incorporation of ^{2}H into the glycerides of the liver and VLDL. In comparing the structure of the

Abbreviations: TG, triacylglycerol; VLDL, very low density lipoprotein; PA, phosphatidic acid; HPLC, high performance liquid chromatography; MG, monoacylglycerol; DG, diacylglycerol; TLC, thin-layer chromatography; CE, cholesteryl ester; FFA, free fatty acid; FC, free cholesterol; PL, phospholipid; PC, phosphatidylcholine; DPNU, dinitrophenylurethane; GLC, gas–liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; UV, ultraviolet; LPL, lipoprotein lipase.

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glycerides in rat VLDL to those in rat liver, methods similar to those that had been used for the studies of the intestine (7) were used. This paper reports data from the structural studies. The results indicate that preexisting hepatic TG are extensively hydrolyzed prior to incorporation into the TG of VLDL. We could not exclude the involvement of the previously demonstrated intestinal 2-MG pathway in the liver. However, the two organs differ as we found only small amounts of hepatic 2-MG and as the activity of the 2-MG acyltransferase in adult rat liver was reported to be low (8). This suggests that any contribution of a 2-MG pathway would be minor. On the other hand, the present data suggest that up to 60% of the TG in VLDL is derived from hepatic TG that is hydrolyzed to *sn*-1,2-diacylglycerol (DG) and then esterified by a pathway that does not involve *sn*-1,2-DG that is synthesized via the PA pathway.

MATERIALS AND METHODS

Materials

Ethyl alcohol-$^2H_6$ (anhydrous) was purchased from MSD Isotopes (Division of Merck Frosst Canada Inc., Montreal, Canada). Alkaline phosphatase and phospholipase C (type I and type V) were obtained from Sigma Chemical Co. (St. Louis, MO), while 3,5-dinitrophenyl isocyanate was from Sumka Chemical Analysis Service LTD (Osaka, Japan). Silica gel G soft layer thin-layer chromatography (TLC) plates were purchased from Fisher Scientific Ltd. (Ottawa, Canada). The BCA protein assay kit was obtained from Pierce Chemical Co. (Rockford, IL) and ethyl magnesium bromide was from Aldrich Chemical Co. (Milwaukee, WI). Somnotal was from M.T.C. Pharmaceuticals, Canada Packer Inc. (Cambridge, Ontario, Canada).

Animals

Male Wistar rats from Charles River Canada (Division of Bausch and Lomb Canada Inc., St-Constant, Quebec), weighing 300 g, were infused with perdeuterated ethanol using a modification of a previously described method (9, 10). The animals were anesthetized with Somnotal (50 mg/kg) and an intraperitoneal cannula was inserted. The infusate, which contained 20% fructose and 10% $^2H_6$-ethanol in normal saline, was infused via a Harvard pump at a rate of 1.1 ml/h. Fructose was given in order to enhance VLDL lipid production (9). After 13 h the rats were killed by drawing blood from the abdominal aorta. The blood was immediately chilled to 4°C and the plasma was separated within 20 min. It was then ultracentrifuged in a Ti 70 rotor (Beckman Instruments, Toronto, Ontario, Canada) 35,000 rpm at 10°C for 18 h in order to obtain VLDL. The livers were immediately removed from the rats and were frozen in liquid nitrogen. They were then extracted with chloroform–methanol 2:1. The total lipid extracts were stored at −20°C under nitrogen.

Separation of lipid classes

The total lipid extracts were resolved into cholesteryl ester (CE), TG, free fatty acid (FFA), diacylglycerol (DG), free cholesterol (FC), and total phospholipid (PL) fractions by TLC on silica gel H plates developed in heptane–isopropyl ether–acetic acid 60:40:4 (7). The PL were extracted from the origin with chloroform–methanol–water–acetic acid 50:39:10:1 and phosphatidylcholine (PC) was isolated by TLC using chloroform–methanol–acetic acid–water 25:15:4:2 as the developing solvent (11).

Isolation of phosphatidic acid (PA)

The hepatic PA was separated by TLC as described previously (7). The total hepatic cellular lipids were resolved by TLC on silica gel H containing 7% magnesium acetate with chloroform–methanol–ammonia–water 65:35:1:3 as the mobile phase. The PA, which migrated just above the origin, was extracted with chloroform–methanol–water–acetic acid 50:39:10:1 and purified in an acidic TLC system using chloroform–methanol–acetone–acetic acid–water 30:10:40:10:5 as mobile phase. PA standard was prepared from egg yolk PC by hydrolysis with cabbage phospholipase D (12).

Isolation and quantitation of free DG

The free *sn*-1,2(2,3)-DG were separated from X-1,3-DG by TLC on silica gel H plates using heptane–isopropyl ether–acetic acid 60:40:4 as described above. The X-1,3-DG comigrated with free cholesterol in this system. The *sn*-1,2(2,3)-DG and X-1,3-DG were quantitated by gas–liquid chromatography (GLC) (7). The *sn*-1,2(2,3)-DG were converted to the 3,5-dinitrophenylurethane (DNPU) derivatives (see below), which were resolved and quantitated by chiral phase HPLC as described previously (7).

Total lipid profiles

The plasma and liver total lipid profiles were determined by GLC after digestion with phospholipase C Type I and Type V, respectively. The digestion converted the PL to the corresponding DG and ceramides, which were trimethylsilylated along with other neutral lipids prior to the GLC analyses (13).

High performance liquid chromatography (HPLC) and mass spectrometry (LC/MS) of TG

The reverse phase HPLC analyses of triacylglycerols were performed with a Hewlett-Packard Model 1050 liquid chromatograph equipped with a Supelcosil LC-18 reverse phase column (25 cm × 0.46 mm ID, Supelco Co., Mississauga, Ontario) coupled to a Varex ELSD II light scattering detector (Varex Co., Burtonsville, MD).

The column was eluted with a linear gradient of 10–90% isopropanol in acetonitrile over 90 min at a flow rate of 1 ml/min. The ELSD detector was operated using a N_2 flow setting of 42 mm and a drift tube temperature of 85°C. The signal was monitored and integrated with a Hewlett-Packard Model 3396A integrator. Reversed phase LC/MS of the triacylglycerols was performed as previously described using a direct liquid inlet interface and positive chemical ionization (14).

Preparation of DG and DNPU derivatives

The *sn*-1,2(2,3)- and X-1,3-DG moieties of TG were prepared by random degradation with the Grignard reagent and were resolved by borate TLC using chloroform–acetone 97:3 as the developing solvent (7). The *sn*-1,2-DG from PC were obtained by phospholipase C digestion of the appropriate TLC fraction (11). The *sn*-1,2-DG from PA were prepared by hydrolysis with alkaline phosphatase as described by Ehle, Muler, and Horn (15). The DG were converted into the DNPU derivatives by reaction with dinitrophenylisocyanate and the DNPU derivatives were purified by TLC on silica gel G using hexane–dichloromethane–ethanol 40:10:3 prior to chiral phase HPLC (7).

Chiral and reversed phase HPLC of enantiomeric-DG

The enantiomeric DG were resolved as the DNPU derivatives on a chiral HPLC column (A-KO3) containing (R)-(+)-1-(1-naphthyl) ethylamine polymer using hexane–dichloromethane–ethanol 40:10:1 as a mobile phase (7). The HPLC was done on Hewlett-Packard Model 1090 Liquid Chromatograph, Series II, interfaced with Hewlett-Packard Model 5989A Quadrupole mass spectrometer using the Hewlett-Packard thermospray interface. The HPLC elution profile was obtained by recording the UV absorption at 254 nm. The molecular species of the DG enantiomers were separated as the DNPU derivatives by LC/MS on reversed phase Supelcosil C-18 column (25 cm × 0.4 cm ID) using a linear gradient of 20–50% isopropanol in methanol over 30 min as the solvent at a flow rate of 1 ml/min. Negative chemical ionization was obtained by post-column addition of methanol–0.2 M ammonium acetate 50:50 at 0.2 ml/min. The negative thermospray ionization was obtained by using the filament-on mode with a fragmenter setting of 100.

Stereospecific analyses

The positional distribution of the fatty acids was calculated from the fatty acid composition in the *sn*-1,2- and *sn*-2,3-DG, the X-1,3-DG and the total TG as described by Yang and Kuksis (7). Other calculations were made to estimate the contributions of various subpools of fatty acids and *sn*-1,2- and *sn*-2,3-DG to the free X-1,2-DG and the VLDL and liver TG pools.

Analysis of fatty acid methyl esters

Gel scrapings containing individual lipid classes and other purified lipid fractions were subjected to acidic methanolysis using 6% H_2SO_4 in methanol at 80°C for 2 h and the fatty acid methyl esters were analyzed by GLC (11).

The analysis was done on a Hewlett-Packard (Palo Alto, CA) Model 5890 Series II capillary gas chromatograph interfaced with a Model 5989A quadrupole mass spectrometer, a Model 98785A computer terminal, and Model 6000/330S data unit. The GC column (12 m × 0.2 mm ID) was coated with HP1 (Hewlett-Packard) cross-linked methyl silicone gum (0.33 μm film thickness). The electron energy was 70 eV. The fatty acid methyl esters were eluted by temperature programming from 100–190°C at 20°C/min and then up to 255°C at 5°C/min.

TABLE 1. Fatty acid composition of PC, of TG, and of *sn*-1,2- and *sn*-2,3-DG prepared from the TG of liver and of plasma VLDL

Fatty Acids	*sn*-1,2-DG		*sn*-2,3-DG		Original TG		PC	
	Liver	VLDL	Liver	VLDL	Liver	VLDL	Liver	VLDL
				%				
14:0	1.6 ± 0.8	1.6 ± 0.2	2.2 ± 0.9	1.0 ± 0.9	2.2 ± 0.8	1.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.0
15:0	0.7 ± 0.3	0.8 ± 0.2	0.3 ± 0.0	0.1 ± 0.4	0.5 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
16:0	37.7 ± 2.2	35.1 ± 0.5	9.8 ± 0.4	13.2 ± 3.5	26.5 ± 0.6	26.0 ± 1.5	25.2 ± 3.9	23.6 ± 1.0
16:1n-7	3.8 ± 1.0	3.1 ± 0.1	5.1 ± 2.3	2.6 ± 2.0	4.4 ± 1.5	2.6 ± 0.3	1.0 ± 0.9	0.7 ± 0.2
17:0	0.4 ± 0.0	0.6 ± 0.2	0.4 ± 0.2	0.1 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
18:0	3.8 ± 0.3	5.0 ± 0.3	6.0 ± 2.3	0.8 ± 0.6	6.2 ± 0.5	3.2 ± 0.3	23.7 ± 1.1	24.0 ± 0.5
18:1n-9	21.2 ± 2.0	18.9 ± 0.2	24.2 ± 2.1	24.7 ± 0.1	19.6 ± 2.2	19.5 ± 0.5	4.5 ± 0.8	4.3 ± 0.3
18:1n-7	2.3 ± 0.3	2.4 ± 0.2	2.6 ± 0.6	2.7 ± 0.0	2.5 ± 0.5	2.5 ± 0.2	2.6 ± 0.5	2.1 ± 0.1
18:2n-6	26.0 ± 2.1	25.7 ± 0.3	30.5 ± 4.8	38.3 ± 3.6	24.2 ± 2.4	30.6 ± 1.2	12.3 ± 0.1	14.8 ± 0.0
20:4n-6	1.0 ± 0.0	2.4 ± 0.4	3.5 ± 1.3	4.8 ± 1.7	2.6 ± 0.5	3.9 ± 1.2	21.1 ± 4.8	21.4 ± 0.8
22:5n-3	0.8 ± 0.1	2.0 ± 0.6	6.9 ± 1.0	3.4 ± 0.7	4.7 ± 0.9	2.9 ± 0.8	0.9 ± 0.2	1.2 ± 0.0
22:6n-3	0.7 ± 0.1	2.5 ± 0.1	8.6 ± 0.6	9.9 ± 1.2	6.1 ± 0.0	6.5 ± 0.7	5.5 ± 1.7	6.7 ± 0.3

The values are average ± range/2 from two rats.

RESULTS

Fatty acids of liver and VLDL lipids

Table 1 gives the quantitative estimates of the relative proportions of the fatty acids in the liver and VLDL TG and PC, and in the enantiomeric *sn*-1,2- and -2,3-DG prepared from the TG of liver and of VLDL. The fatty acid composition of the *sn*-1,2-DG prepared from the liver TG is very similar to that prepared from VLDL TG. The corresponding *sn*-2,3-DG moieties, however, differ in the content of 16:0, 18:0, and 18:2. The whole TG in the present study has a higher proportion of 16:0 and lower proportion of 18:1 than previously reported (16). The fatty acid composition of liver and VLDL PC were quite similar, but had less 18:1 and more 20:4 than previously reported (16). As the fatty acids of PC are in the *sn*-1 and *sn*-2 positions, the PC fatty acid composition was compared to that of *sn*-1,2-DG prepared from each of liver TG and VLDL TG. Table 1 shows them to be very different.

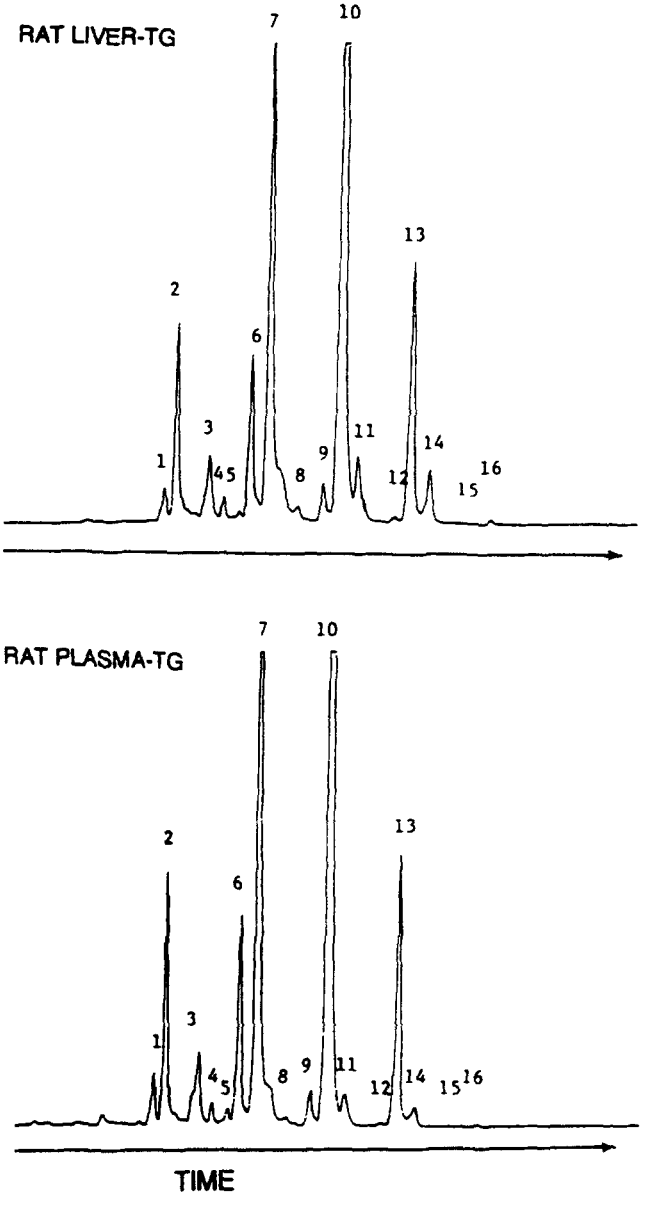

Fig. 1. Reversed phase HPLC profiles of rat liver (top) and VLDL (bottom) triacylglycerols. The peak numbering in this figure and Table 2 corresponds to each other. HPLC conditions: Hewlett-Packard Model 1050 liquid chromatograph equipped with a Supelcosil LC-18 reverse phase column (25 cm × 0.46 mm ID), coupled to a Varex ELSD II light scattering detector; solvent, a linear gradient of 10–90% isopropanol in acetonitrile over 90 min at flow rate of 1 ml/min.

TABLE 2. Reversed phase HPLC separation of liver and plasma TG

Peak No.[a]	TG Major Species	Peak Area[b] Liver	Plasma
		%	
1	18:1-18:2-22:6	2.7	3.2
2	16:0-18:2-22:6	7.8	8.6
3	18:1-18:2-20:4	6.1	6.3
4	16:0-16:0-22:6	2.1	1.7
5	18:1-18:1-22:6	1.3	1.8
6	18:1-18:2-18:2	7.5	8.1
7	16:0-18:2-18:2	16.5	20.4
8	16:0-18:1-20:4	5.0	1.0
9	18:1-18:1-18:2	3.2	2.7
10	16:0-18:1-18:2 + 18:0-18:2-18:2	25.4	29.5
11	16:0-16:0-18:2	5.1	3.0
12	18:1-18:1-18:1	1.1	0.6
13	16:0-18:1-18:1 + 18:0-18:1-18:2	10.4	10.0
14	16:0-16:0-18:1	4.2	2.3
15	18:0-18:1-18:1	0.6	0.3
16	16:0-18:0-18:1	0.8	0.6

[a] The peak numbers correspond to those in Fig. 1.
[b] HPLC conditions are given under Methods. Peak areas are averages of two repeat injections, as obtained using a light scattering detector.

Molecular species of liver and VLDL TG

Figure 1 shows reversed phase HPLC profiles of TG from the liver and from the plasma VLDL of a rat. The molecular species of TG corresponding to each peak was identified by reversed phase LC/MS and given in **Table 2.** Table 2 also shows the relative quantity of these TG species in the liver and VLDL lipids. Although the elution profiles appear closely similar, quantitative differences can be seen, especially in the higher content of 16:0-18:2-18:2 (peak 7), 16:0-18:1-18:2 + 18:0-18:2-18:2 (peak 10), and lower content of 16:0-18:1-20:4 (peak 8), 16:0-16:0-18:2 (peak 11) and 16:0-16:0-18:1 (peak 14) species in plasma VLDL compared to liver TG. Comparable differences between plasma and liver TG were obtained for other rats.

Stereospecific analyses of liver and VLDL TG

Figure 2 shows the chiral phase HPLC separation of the *sn*-1,2-DG and the *sn*-2,3-DG prepared by Grignard

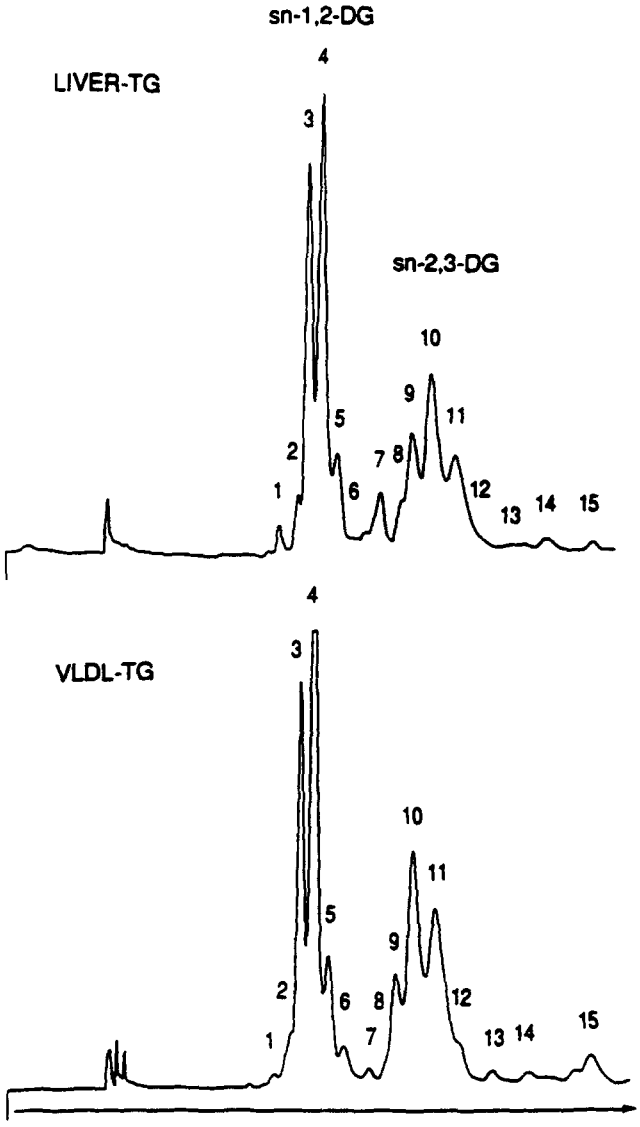

Fig. 2. Chiral phase HPLC separation of *sn*-1,2-DG and *sn*-2,3-DG prepared from the TG of the liver and of the VLDL of one rat. Peaks 1–6 are the *sn*-1,2-enantiomers; peaks 7–15 are the *sn*-2,3-enantiomers. The instrumental and experimental conditions were as described in text.

degradation of the TG from the liver and from the VLDL of a rat. When run as the DNPU derivatives, the *sn*-1,2-DG are eluted well ahead of the *sn*-2,3-DG (peaks 1–6 and peaks 7–15, respectively). The peaks corresponding to each enantiomer were collected and an aliquot was used to determine the fatty acid composition, which was shown in Table 1. Previously described methods (7) were used to calculate the fatty acid composition and positional distribution in the *sn*-1,2-DG and *sn*-2,3-DG moieties of the TG and in the entire TG of VLDL and liver from this data. **Table 3** shows the differences in the positional distribution of the fatty acids in the TG of the liver and VLDL. The major fatty acid at *sn*-1 position of both liver TG and VLDL TG was 16:0. The relative proportions of different fatty acids at the *sn*-1 position of liver TG were the same as those in VLDL TG. The major fatty acids at *sn*-2 position of liver and VLDL TG were 18:1 and 18:2. Again, at position 2 of both liver and VLDL TG, except for 22:6n–3, there were similar proportions of each fatty acid. That particular fatty acid (22:6n–3) represented 5.4% of the fatty acids at the *sn*-2 position of VLDL-TG. However, it only represented 0.1% of the fatty acids at the same position of liver TG. The strong similarities in the fatty acid compositions of liver and VLDL TG at positions *sn*-1 and *sn*-2 suggested that most of the VLDL TG could have been derived via *sn*-1,2-DG from liver TG. By contrast, there were major differences between liver TG and VLDL TG in the fatty acid composition at the *sn*-3 position. This suggested that if liver TG was a source of VLDL TG, it would have to be greatly modified in the *sn*-3 position. Hence, the TG in VLDL could not be obtained from unaltered hepatic TG.

Analyses of molecular species of enantiomeric DG

Figure 3 shows that in addition to similarities in composition and positional distribution there were also close similarities in the molecular association of the fatty acids in the *sn*-1,2-DG moieties prepared from TG of the liver and of the VLDL. The species are identified by the acyl carbon:double bond number, with the ion abundances indicated for the major peak in each plot. **Table 4** gives the composition of the major molecular species of the enantiomeric DG as calculated from the areas of the single ion plots obtained from LC/MS. With a few exceptions, the compositions of the corresponding *sn*-1,2-enantiomers are similar. Similar LC/MS methods were used to compare the molecular species profiles for the *sn*-2,3-DG moieties of the corresponding VLDL and liver TG. Table 4 also shows that the differences between the *sn*-2,3-DG of the liver TG and of VLDL TG, anticipated on basis of differences in the fatty acid composition of the *sn*-3 position (Table 3), have been partly masked by the common fatty acids in the *sn*-2 position of the *sn*-2,3-DG.

Composition of liver *sn*-1,2-free DG, PA, TG, and PC

Figure 4 shows the chiral phase separation of the free DG (not prepared from TG) of the liver into the *sn*-1,2- and *sn*-2,3-enantiomers. It was estimated that 10% of free DG was *sn*-2,3-DG. **Figure 5** shows the reverse phase HPLC separation of the molecular species of the *sn*-1,2-DG prepared from hepatic PA and of the free hepatic *sn*-1,2-DG. The identity and quantitative composition of these *sn*-1,2-DG moieties is given in **Table 5.** The free *sn*-1,2-DG was considerably richer than the PA in the shorter chain and more unsaturated species (peaks from 1 to 6). The marked differences between the PA and free *sn*-1,2-DG indicate that the free DG was not made exclusively by hydrolysis of PA.

TABLE 3. Positional distribution of fatty acids in liver and plasma VLDL-TG

Fatty Acids	Liver-TG			VLDL-TG		
	*sn*1	*sn*2	*sn*3	*sn*1	*sn*2	*sn*3
	%					
14:0	2.1 ± 0.6	1.1 ± 1.0	3.3 ± 0.2	2.0 ± 1.1	1.2 ± 0.7	0.7 ± 0.6
15:0	1.1 ± 0.3	0.2 ± 0.3	0.3 ± 0.4	1.4 ± 0.4	0.2 ± 0.1	0.0 ± 0.4
16:0	59.9 ± 0.8	15.6 ± 3.6	4.0 ± 3.1	51.8 ± 1.7	18.4 ± 0.7	8.0 ± 4.3
16:1n-7	3.0 ± 0.0	4.6 ± 2.0	5.6 ± 1.3	2.8 ± 2.1	3.4 ± 2.3	1.7 ± 0.5
17:0	0.5 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	1.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.2
18:0	6.8 ± 2.1	0.9 ± 2.7	11.0 ± 0.5	11.2 ± 0.1	- 1.2 ± 0.5	0.5 ± 1.3
18:1n-9	10.2 ± 1.6	32.2 ± 2.4	16.3 ± 0.6	9.0 ± 0.9	28.7 ± 0.5	20.7 ± 0.6
18:1n-7	2.4 ± 0.1	2.3 ± 0.5	3.0 ± 0.4	2.2 ± 0.4	2.5 ± 0.1	2.8 ± 0.1
18:2n-6	11.6 ± 1.7	40.3 ± 5.9	20.7 ± 0.9	15.2 ± 2.6	36.1 ± 2.0	40.5 ± 3.1
20:4n-6	0.7 ± 0.8	1.2 ± 0.8	5.7 ± 1.0	1.9 ± 0.2	2.9 ± 1.1	6.8 ± 3.5
22:5n-3	0.4 ± 0.4	1.2 ± 0.6	12.5 ± 2.0	1.9 ± 0.8	2.2 ± 0.5	4.7 ± 0.5
22:6n-3	1.3 ± 1.0	0.1 ± 1.1	17.0 ± 0.2	0.3 ± 0.2	5.4 ± 0.4	14.5 ± 1.2

The values are average ± range/2 from two rats.

The total cellular lipids contained 26% of TG, 0.19% of free DG, and less than 0.07% of PA. On the basis of the mass analyses of the molecular species of the enantiomeric liver glycerolipids it was possible to recognize several possible sources for hepatic free DG. Thus, a subtraction of the *sn*-1,2-DG moieties of PA from the *sn*-1,2-DG moieties of the free DG in Table 5 indicated that a maximum of 40% of the free DG could have been derived from hydrolysis of PA by the phosphohydrolase. The remainder of free *sn*-1,2-DG (60%) could be accounted for in large part by the *sn*-1,2-DG released from liver TG by hydrolysis. The extra 20:4 containing species in the free DG could have been used for PL synthesis or originated from hepatic PC hydrolysis (see Fig. 6A). **Figure 6A** shows that the fatty acid composition of liver free *sn*-1,2-DG lies between those of the *sn*-1,2-DG of PA and liver-TG, indicating that the free *sn*-1,2-DG is a mixture of the hydrolytic products of both PA and liver-TG.

Composition of *sn*-2,3-DG of free DG and liver and VLDL TG

Figure 6B compares the fatty acid composition of the *sn*-2,3-DG moieties of free DG and those prepared from the TG of VLDL and liver. There are marked similarities between the *sn*-2,3-DG of free DG and those derived chemically from liver TG. The *sn*-2,3-DG prepared from VLDL TG had considerably more 18:2 and less 18:0 than either of the other *sn*-2,3-DG.

DISCUSSION

The origin of VLDL TG is complex and not very well understood (17, 18). This paper reports structural analysis of the glycerides of the liver in comparison to those of VLDL. The data provide new insight into the origin of VLDL TG.

Sources of fatty acids in VLDL-TG

The fatty acids in VLDL TG may be derived from de novo hepatic synthesis or from preformed sources, such as plasma FFA, lipoprotein TG fatty acids, or TG stored in the liver. The relative importance of each source is not known. Arbeeny et al. (19) blocked new FA synthesis by inhibiting acetyl CoA carboxylase and found a major decrease in VLDL-TG production, suggesting that the biosynthesis of fatty acids is critical to VLDL-TG production. Steiner and Ilse (20) have shown in dogs that 15% of VLDL TG fatty acids are derived from plasma FFA. Park et al. (21) have reported that de novo synthesized FA in the rat can account for anywhere between 3 and 30% of the TG fatty acids of VLDL. Wiggins and Gibbons (6), using radioactive oleate and glycerol, have estimated that over 70% of the VLDL TG is derived from hydrolysis of hepatic stored TG and subsequent reesterification. This conclusion is supported by earlier qualitative work (1–5), which had shown that newly formed hepatic TG are not directly secreted but first enter a cytoplasmic storage pool and then are hydrolyzed and reesterified prior to incorporation into plasma VLDL.

The present study shows that the fatty acid composition (Table 1) and major molecular species (Table 2) of TG in the liver were very similar to those of the TG in VLDL. In addition, closely similar LC/MS profiles were obtained for the corresponding *sn*-1,2-DG moieties of the TG from both sources (Table 4). The two types of TG, however, differed in their *sn*-2,3-DG moieties. This difference was mainly due to a higher proportion of 18:2 and lower proportion of 18:0 fatty acids in the *sn*-3 position of hepatic TG as compared to VLDL TG. Although some

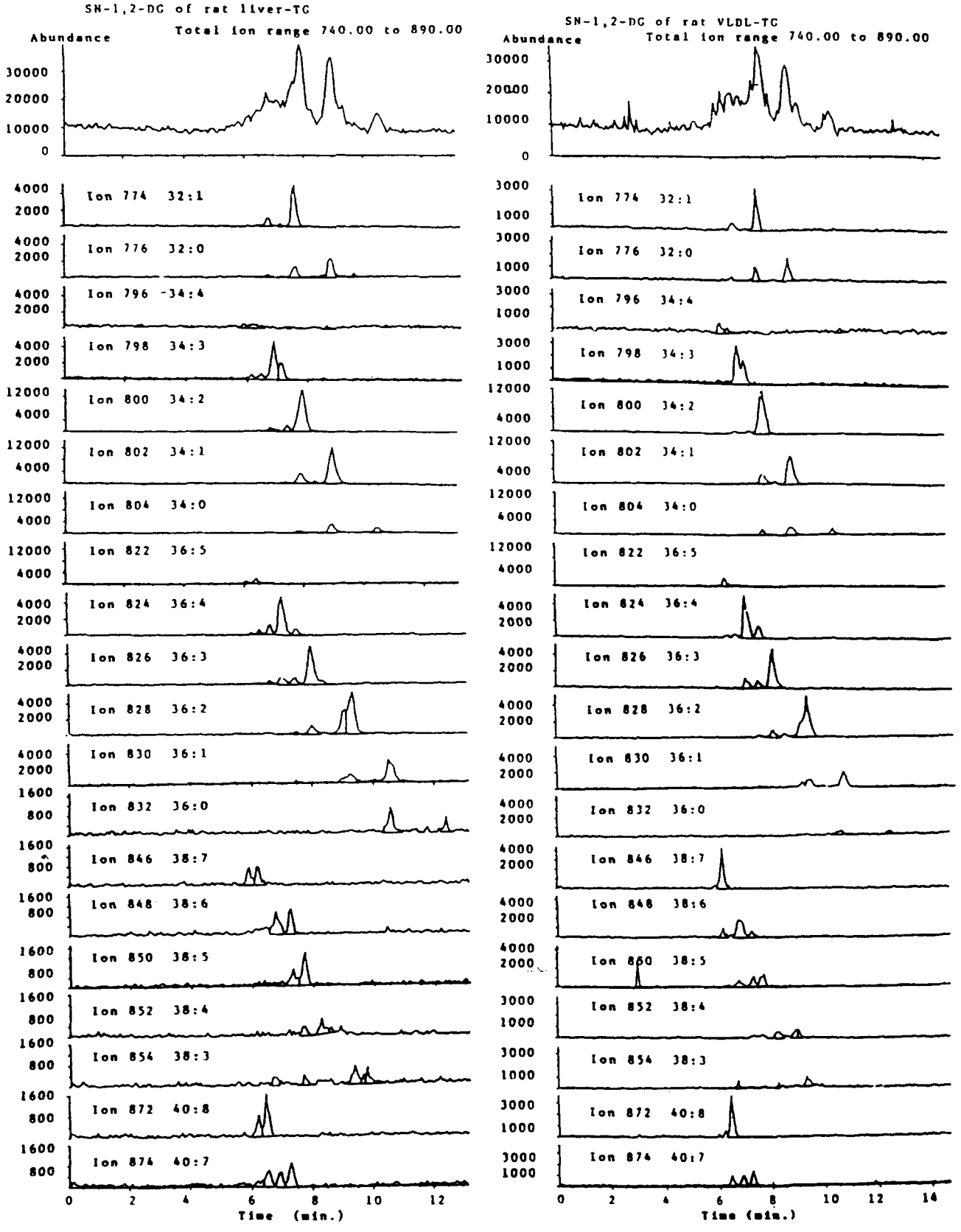

Fig. 3. Reversed phase LC/MS profiles of the *sn*-1,2-diacylglycerols of rat liver and plasma VLDL TG. Top panel: ion sum from *m/z* 740 to 890 versus elution time. Lower panels: single ion mass chromatograms corresponding to the $[M-H]^-$ ions of various molecular species indicated by carbon number:double bond number. The panels on the left show *sn*-1,2-DG prepared from liver TG. Those on the right show *sn*-1,2-DG prepared from VLDL TG. LC/MS conditions: Hewlett-Packard Model 1090 liquid chromatograph equipped with a Supelcosil LC-18 reversed phase column (25 cm × 0.46 mm ID) interfaced with a Hewlett-Packard Model 5989A Quadrupole mass spectrometer using a Hewlett-Packard thermospray interface. Mass spectra acquired using the filament-on negative thermospray mode. Other conditions as given in text.

TABLE 4. Major species of enantiomeric DGs from liver and plasma VLDL-TG

			Liver TG		VLDL-TG	
m/z	C#/DB	DG Species	*sn*1,2	*sn*2,3	*sn*1,2	*sn*2,3
			%			
774	32:1	16:0-16:1	6.1 ± 0.4	1.4 ± 0.1	3.5 ± 0.2	0.8 ± 0.1
776	32:0	16:0-16:0	3.0 ± 0.0	1.1 ± 0.0	2.2 ± 0.2	1.3 ± 0.1
796	34:4	14:0-20:4	1.3 ± 0.4	2.0 ± 0.0	0.8 ± 0.2	1.2 ± 0.3
798	34:3	16:1-18:2	5.6 ± 0.2	3.5 ± 0.1	4.8 ± 1.3	2.1 ± 0.4
798	34:3	16:0-18:3	1.2 ± 1.2	0.0 ± 0.0	1.5 ± 1.5	0.0 ± 0.0
800	34:2	16:0-18:2	26.1 ± 2.6	2.2 ± 0.1	22.6 ± 0.2	1.2 ± 0.0
800	34:2	16:1-18:1	0.0 ± 0.0	4.8 ± 0.1	0.0 ± 0.0	3.5 ± 0.2
802	34:1	16:0-18:1	19.5 ± 0.4	4.7 ± 0.1	16.4 ± 1.9	3.3 ± 0.4
804	34:0	16:0-18:0	2.7 ± 0.2	1.2 ± 0.1	2.1 ± 0.1	1.2 ± 0.1
822	36:5	16:0-20:5	1.9 ± 0.3	3.8 ± 0.6	2.5 ± 0.1	3.5 ± 0.1
824	36:4	18:2-18:2	5.3 ± 0.0	14.3 ± 0.3	5.1 ± 0.1	12.4 ± 0.3
824	36:4	16:0-20:4	1.1 ± 0.3	0.3 ± 0.3	2.0 ± 0.7	1.5 ± 0.1
826	36:3	18:1-18:2	6.4 ± 0.6	16.1 ± 0.4	6.5 ± 0.2	15.7 ± 0.7
828	36:2	18:1-18:1	2.8 ± 0.3	8.8 ± 0.4	2.4 ± 0.3	8.0 ± 2.0
828	36:2	18:0-18:2	4.7 ± 0.2	2.8 ± 0.0	4.6 ± 1.0	1.6 ± 1.0
830	36:1	18:0-18:1	4.2 ± 0.5	2.2 ± 0.1	3.7 ± 0.4	1.9 ± 0.1
832	36:0	18:0-18:0	0.9 ± 0.3	0.0 ± 0.0	0.4 ± 0.1	0.1 ± 0.1
846	38:7	16:1-22:6	0.5 ± 0.2	4.0 ± 0.4	4.0 ± 0.2	7.1 ± 0.4
848	38:6	16:0-22:6	1.0 ± 0.2	4.5 ± 0.1	3.3 ± 0.5	6.8 ± 0.2
850	38:5	16:0-22:5	1.4 ± 0.1	2.1 ± 0.3	1.7 ± 0.0	2.2 ± 1.0
850	38.5	18:1-20:4	0.0 ± 0.0	1.1 ± 0.2	0.6 ± 0.6	1.5 ± 1.5
852	38:4	18:0-20:4	0.6 ± 0.1	1.6 ± 0.0	0.9 ± 0.1	1.3 ± 0.2
854	38:3	18:0-20:3	0.7 ± 0.1	1.7 ± 0.3	1.3 ± 0.3	1.6 ± 0.3
872	40:8	18:2-22:6	1.5 ± 0.0	5.2 ± 0.1	3.7 ± 0.1	8.5 ± 0.2
874	40:7	18:1-22:6	0.6 ± 0.0	3.6 ± 0.5	0.9 ± 0.1	2.9 ± 0.1
874	40:7	18:2-22:5	0.7 ± 0.3	3.0 ± 0.2	1.5 ± 0.2	4.6 ± 0.3
876	40:6	18:0-22:6	0.2 ± 0.2	2.5 ± 0.1	0.4 ± 0.0	2.8 ± 0.3
878	40:5	18:0-22:5	0.2 ± 0.2	1.6 ± 0.2	0.6 ± 0.2	1.7 ± 0.1

The DG species were identified by reversed LC/MS; *m/z*, mass/charge ratio of the molecular ion; C#/DB, carbon number/double bonds. The values are average ± range/2 from two rats.

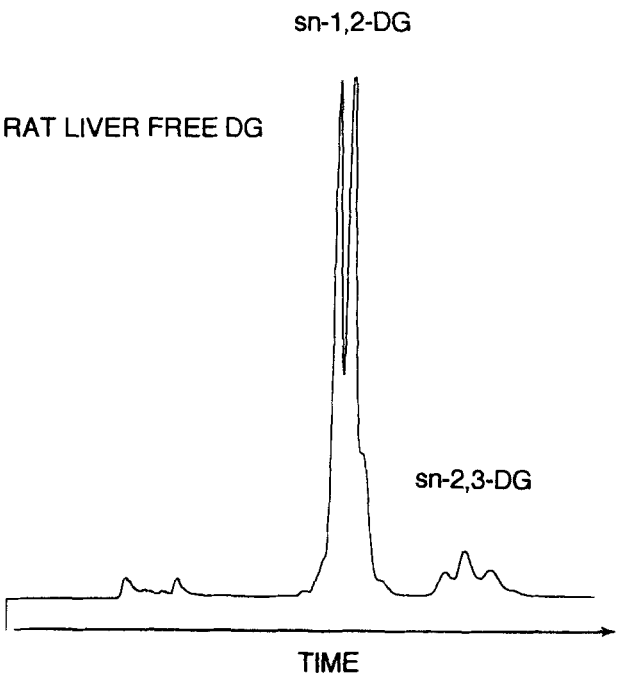

Fig. 4. Chiral phase HPLC separation of the free *sn*-1,2- and -2,3-diacylglycerols of rat liver. Chromatographic conditions as in Fig. 2.

might postulate that the difference between liver-TG and VLDL-TG could be due to modification of VLDL-TG occurring after its secretion, we consider this to be unlikely. The major extrahepatic modification of VLDL-TG would be due to its hydrolysis by lipoprotein lipase (LPL). Wang, Hartsuck, and McConathy (22) reported that LPL preferentially hydrolyzed TG fatty acids in the order of 18:1 > 18:2 > 14:0 > 16:0 > 18:0. Such hydrolysis of VLDL-TG would not produce the compositional differences between liver-TG and VLDL-TG that we observed. Therefore, the present data are more consistent with previous suggestions (3) that TG stored in liver is not incorporated en bloc into VLDL.

PA as a source of VLDL-TG

If VLDL TG were to be directly derived from de novo fatty acid synthesis or complete hydrolysis of hepatic TG, its fatty acids might appear in PA and free DG as intermediates during the esterification process. Under these circumstances, the fatty acid and molecular species composition of the hepatic PA, the hepatic free *sn*-1,2-DG and the *sn*-1,2-DG moiety of VLDL TG should all be similar. This was not the case. These findings effectively excluded a direct contribution to VLDL-TG formation by the path-

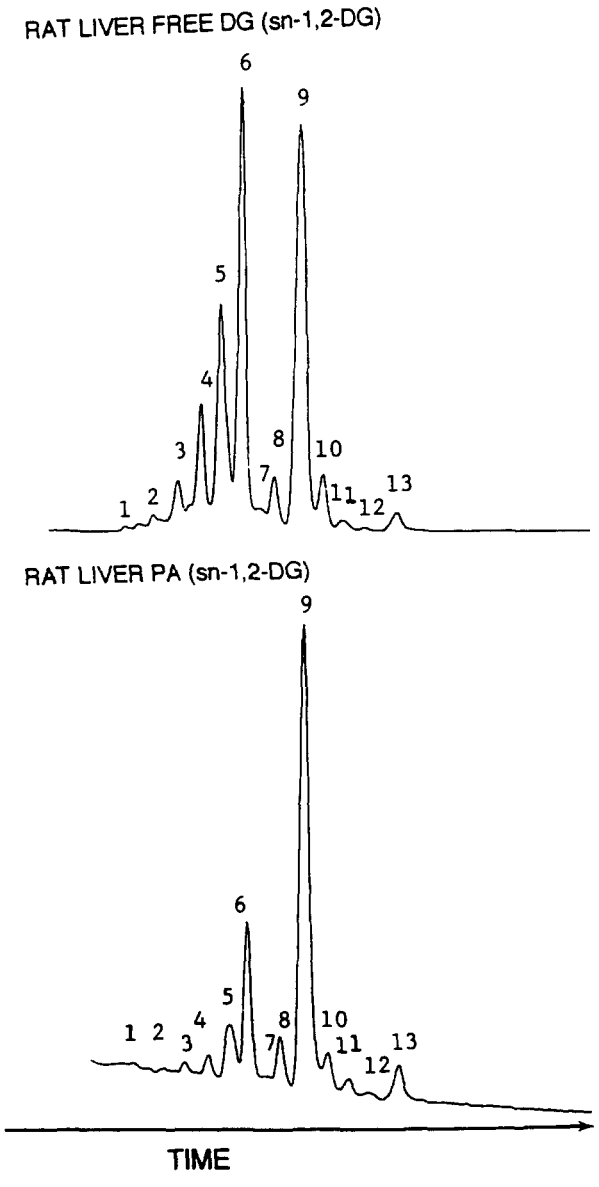

Fig. 5. Reversed phase HPLC profiles of the *sn*-1,2-diacylglycerol moieties of rat liver phosphatidic acid and free diacylglycerols. The peak identification is given in Table 5. The HPLC column is as in Fig. 1. Solvent condition: 20–50% isopropanol in methanol over 30 min at a flow rate of 1 ml/min. Other chromatography conditions are as given in the text.

way generally believed to be responsible for all TG biosynthesis in the liver (23).

MG pathway for VLDL-TG formation

The 2-MG are common end products of TG hydrolysis and reesterification of 2-MG to TG is a major pathway of biosynthesis of chylomicron TG in the intestine (7). A comparison of the fatty acid composition of the *sn*-2 position of the liver and VLDL TG indeed revealed very close similarity, and it was estimated that the MG pathway could have theoretically accounted for 80% of the VLDL TG. *Sn*-2-MG were not recovered from the lipid extracts of the liver. However, as the liver contains a nonspecific MG hydrolase (24), their transient presence in a quantitatively small, rapidly turning over pool could not be excluded. Even if such a pool existed, the likelihood of reesterification of the products of hydrolysis of hepatic TG for secretion as VLDL TG via the MG pathway is small. This is particularly so as the activity of 2-MG acyl transferase in adult rat livers is very low (1/700th that of suckling rat livers) (8).

DG pathway of VLDL formation

A more likely pathway of VLDL TG formation might involve a transacylation of the DG released upon hydrolysis of cytoplasmic TG. This would also retain the fatty acid composition of the *sn*-2 position of the liver TG and permit extensive acyl exchange at the *sn*-1 and *sn*-3 position of free DG. A recently described DG-transacylase (25), however, must be discounted as it yields an equivalent amount of 2-MG for each TG molecule formed.

The DG acyltransferase associated with the PA pathway is located on the cytoplasmic side of the microsomal membrane (26, 27) and would again release the TG to cytoplasmic storage. Therefore, if a DG pathway were to be a major mechanism of VLDL TG formation, it would require that the hydrolysis of the liver TG stops at the X-1,2-DG stage. A TG lipase, which attacks preferentially primary esters, has been described for the intestine by Rao and Mansbach (28). Whether a similar lipase exists in the liver is not yet known. Xia and Coleman (29) have characterized a DG lipase from the neonatal rat liver, but it is not known if it occurs also in adult liver. The presence of TG lipase of unknown stereospecificity in the liver lysosomes has been well established but its inhibition by chloroquine apparently does not block the hydrolysis of liver TG (6). The present finding of *sn*-2,3-DG as 10% of the hepatic free DG suggests that the candidate lipase would lack stereospecificity. As much as 19–25% of hepatic free DG has been previously reported by Akesson (30), who also noted that the *sn*-2,3-DG were more unsaturated than the *sn*-1,2-DG derivatives, both findings being consistent with the present results. In subsequent studies, Akesson, Elovson, and Arvidson (31) showed that *sn*-2,3-DG can be acylated to TG in vitro. The polyunsaturated species of *sn*-1,2-DG moieties could have come from a reversal of PC synthesis, as suggested by Akesson et al. (30, 31). Kahno and Ohno (32) have shown the reversibility of the reaction catalyzed by CDP choline:*sn*-1,2-DG choline phosphotransferase of rat liver microsomal preparations and have demonstrated the incorporation of the released DG into liver TG.

Proposed mechanism of VLDL-TG formation

In view of the above discussion, the similarity in the

TABLE 5. Comparison of *sn*-1,2-diacylglycerol species of liver PA, free DG, TG, and PC from reverse phase HPLC separation

Peak #	DG species	PA[a]	Free DG[a]	TG[b]	PC[b]
			%		
1	UNK	0.2 ± 0.0	0.9 ± 0.0	ND	0.4 ± 0.0
2	16:1-20:4 + 18:2-22:6	0.7 ± 0.2	2.1 ± 0.2	1.9 ± 0.3 1.5 ± 0.0	2.4 ± 0.4 0.9 ± 0.0
3	14:0-18:2 + 16:1-18:2 + 16:0-20:5	2.0 ± 0.2	4.4 ± 0.3	ND 5.6 ± 0.2 1.9 ± 0.3	0.9 ± 0.1 1.0 ± 0.1 2.0 ± 0.1
4	16:0-22:6 + 18:1-22:6 + 18:2-18:2	5.4 ± 0.1 ND	9.1 ± 0.1 ND	1.0 ± 0.2 0.6 ± 0.0 5.3 ± 0.0	8.6 ± 0.4 0.7 ± 0.0 1.7 ± 0.5
5	18:1-20:4 + 16:0-20:4 + 14:0-18:1	ND 8.4 ± 1.4 ND	ND 17.4 ± 1.5 ND	ND 1.1 ± 0.3 6.1 ± 0.4	2.8 ± 0.2 11.6 ± 0.6 0.4 ± 0.1
6	16:0-18:2 + 18:1-18:2	16.1 ± 0.2 ND	24.1 ± 0.6 ND	26.1 ± 2.6 6.4 ± 0.6	10.6 ± 0.4 3.0 ± 0.7
7	16:0-22:5	1.1 ± 0.2	2.0 ± 0.3	1.4 ± 0.1	2.0 ± 0.1
8	18:0-22:6	8.9 ± 1.3	3.6 ± 0.2	0.2 ± 0.2	8.2 ± 1.4
9	18:0-20:4 + 16:0-18:1 + 16:0-16:0	48.0 ± 0.8 ND	29.9 ± 1.2 ND	0.6 ± 0.1 19.5 ± 0.4 3.0 ± 0.0	12.3 ± 0.5 2.9 ± 0.1 0.6 ± 0.1
10	18:0-18:2 + 18:1-18:1	4.0 ± 0.0	3.6 ± 0.3	4.7 ± 0.2 2.8 ± 0.3	11.0 ± 0.1 ND
11	UNK	1.7 ± 0.0	1.0 ± 0.0	ND	1.4 ± 0.0
12	UNK	0.5 ± 0.0	0.4 ± 0.0	ND	0.2 ± 0.0
13	16:0-18:0 + 18:0-18:1	3.1 ± 0.0	1.5 ± 0.2	2.7 ± 0.2 4.2 ± 0.5	0.6 ± 0.1 3.8 ± 0.2

The values are average ± range/2 from two rats; UNK, unknown; ND, not detected.
[a]Quantitated by reverse phase HPLC and UV detector.
[b]Quantitated by reverse phase HPLC/mass spectrometry.

composition of the fatty acids in the *sn*-2 position and in molecular species of the *sn*-1,2-DG moieties between the liver and VLDL-TG could be best accounted for by the action of a DG acyltransferase not associated with the PA pathway. The VLDL-TG synthesis could then proceed largely via acylation of the *sn*-1,2-DG released from the TG stored in the cytoplasm. While there is evidence that X-1,2-DG can readily cross the lipid bilayer of a biological membrane (33), the acyl CoA necessary for TG formation cannot (34). In view of this and the demonstration that DG acyltransferase is located on the cytoplasmic side of the microsomal membrane, it would appear that the resynthesis of TG occurs on the cytoplasmic side of the microsome. This would then be followed by transfer of the TG to the microsomal lumen by the TG transfer protein (35). A DG acyltransferase integrated with the TG-rich particles isolated from a novel rough endoplasmic reticulum (RER) subfraction of rat liver has been recently reported by others (18). This TG particle is also assembled independent of the apoB pathway and it has been predicted that it would fuse with a small primordial apoB-containing particle to form nascent VLDL (36). A confirmation of the proposed mechanism requires the isolation of the hepatic TG lipase that attacks the stored TG yielding largely *sn*-1,2-DG and the demonstration that these DG are incorporated into the TG-rich particles of smooth endoplasmic reticulum (SER) lacking apoB.

These studies were performed with funds from the Medical Research Council of Canada. L-Y.Y. is a Fellow of the Canadian Diabetes Association.

Manuscript received 29 April 1994 and in revised form 11 July 1994.

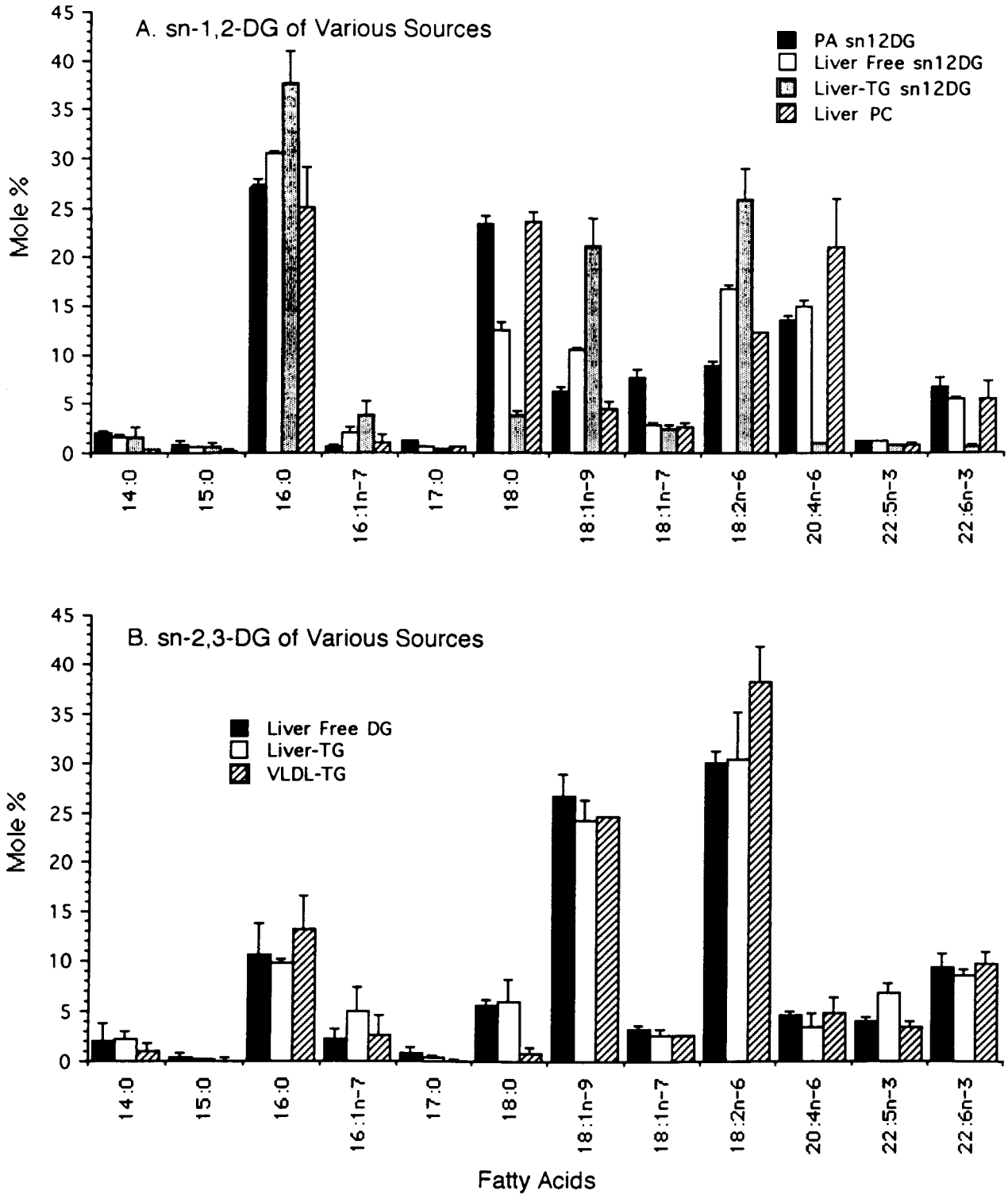

Fig. 6. A: Comparison of fatty acid composition of *sn*-1,2-DG of PA, free DG, TG, and PC from liver. B: Comparison of fatty acid composition of *sn*-2,3-DG from liver free DG, liver TG, and VLDL TG. Each bar represents the average ± range/2 from two animals.

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