Elevation of liver diacylglycerols and molecular species of diacylglycerols in rats fed a lipogenic diet

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Abstract The concentration of diacylglycerols in rat liver was measured by a newly developed procedure that entailed *a*) freeze-clamping of the liver in situ, *b*) lipid extraction with dimyristoylglycerol as an internal standard, *c*) thin-layer chromatography, and *d*) gas–liquid chromatography. Molecular species of diacylglycerol and total diacylglycerols were quantified. The average level of diacylglycerol in livers of chow-fed rats was 102 μg/g wet wt, equivalent to about 180 nmol/g wet wt. A high-carbohydrate fat-free diet, known to increase the rate of fatty acid synthesis, greatly increased the liver diacylglycerol concentration relative to the level observed in rats fed laboratory chow. Diacylglycerol molecular species that contained 16-carbon fatty acids were most markedly elevated. Liver triacylglycerol, free cholesterol, and esterified cholesterol were concurrently increased. Molecular species of triacylglycerols and cholesteryl esters that contained 16-carbon fatty acids were elevated to the greatest degree. The concentrations of total triacylglycerol and cholesteryl esters exhibited a high correlation in the livers of all animals studied, suggesting their coexistence in metabolic pools, predominantly the cores of lipid droplets and newly assembled very low density lipoprotein particles. The correlation of liver diacylglycerol and triacylglycerol contents in the chow-fed rats suggests that the diacylglycerol concentration may be a rate-determining factor in triacylglycerol synthesis when diacylglycerol levels are in the observed range of 70–150 μg/g wet wt. In conclusion, when the rates of fatty acid synthesis and hence triacylglycerol synthesis are increased in the liver, the steady state concentrations of diacylglycerols are also elevated. The tissue diacylglycerol assay described herein should be useful in studying diacylglycerol metabolism, inasmuch as molecular species containing 32, 34, 36, 38, and 40 carbons in the fatty acyl chains are resolved. — **Ontko, J. A., and C-S. Wang.** Elevation of liver diacylglycerols and molecular species of diacylglycerols in rats fed a lipogenic diet. *J. Lipid Res.* 1989. **30:** 691–699.

Supplementary key words: cholesterol • cholesteryl ester • fat-free diet • fatty acid synthesis • fatty acyl-CoA desaturase • gas–liquid chromatography • palmitic acid • sucrose • triacylglycerol

It is well established that diets that contain high levels of carbohydrate and low levels of fat promote hepatic lipogenesis (1–9). Under these nutritional conditions acetyl-CoA carboxylase is activated and carbon flux from citrate to long chain fatty acids via citrate lyase, acetyl-CoA carboxylase, and fatty acid synthetase is enhanced. Esterification of the newly synthesized long chain fatty acids concurrently promotes synthesis, deposition, and secretion of triacylglycerol by the liver (8–13). Since diacylglycerols are intermediates in the synthesis of triacylglycerols (14), it was of interest to examine the liver diacylglycerol concentrations in this state of enhanced flux through this pathway. Diacylglycerols are also messengers in the actions of hormones that initiate phosphatidylinositol hydrolysis (15–18). In this regulatory cascade diacylglycerols activate protein kinase C and much attention has been focused on this process. Methodology was therefore developed for the quantitative assay of liver diacylglycerols and molecular species thereof. Freeze-clamping of the liver in situ was used to prevent changes that might occur following removal of organ samples. The lipids were then extracted and chromatographically resolved. The liver diacylglycerol concentration was found to be markedly affected by the lipogenic diet. This procedure may be useful in the analysis of diacylglycerols in other tissues and biological systems.

EXPERIMENTAL PROCEDURES

Animals

Male Holtzman rats were obtained from Sasco. All animals (200–300 g) were maintained on Purina Laboratory Chow and water ad libitum and were housed at constant temperature (22°C, with lights on at 6:00 AM and off at

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

6:00 PM). The rats were divided into two groups with 12 animals in each group. The control group was continued on the pelleted chow diet and the other group was fed a pelleted fat-free diet (901683, ICN Nutritional Biochemicals, Cleveland, OH). This diet contained 21.1% casein, 16.45% alphacel, 54.45% sucrose, 4.0% salt mixture U.S.P. XIV, plus a complete vitamin mixture (6 g/kg diet). The rats were given this diet ad libitum for 10 days. Animals were not fasted prior to blood and liver removal, since effects of the lipogenic diet on liver lipids were of major interest.

Tissue sampling

Blood and liver samples were obtained between 9:00 AM and 9:30 AM. Six animals (three chow-fed and three fed the lipogenic diet) were processed on each of 4 different days. The diets were scheduled so that each group of rats consumed their respective diets for 10 days.

The tissue clamps were first cooled in liqid nitrogen in a Dewar flask. The rats were individually anesthetized with diethyl ether in a closed chamber. The rats were then placed on a surgery board with continued ether inhalation via a 100-ml beaker which contained cotton saturated with ether. The abdominal cavity was opened, the liver was exposed, a blood sample was quickly removed from the vena cava, the animal was inverted, and a portion (approximately 2 g) of the median lobe was freeze-clamped in situ with aluminum clamps at − 196°C (19–21). These clamps were constructed of two symmetrical polished aluminum cylinders, each of which was 10.2 cm in diameter and 2.0 cm thick. The stainless steel handles were 66 cm long with the fulcrum 48.3 cm from the end of the handle. The liver samples were instantly frozen in a flat cake approximately 0.5 mm thick and placed in a bucket of crushed dry ice (solid CO_2). We have previously used this procedure to instantaneously arrest metabolism prior to the processing of heart (22) and liver (23, 24) for the assay of metabolic intermediates. The remainder of each liver, following freeze-clamping, was removed from the animal to determine the residual liver weight. This was added to the weight of the freeze-clamped sample (described below) to provide total liver weight.

Lipid extraction

The frozen liver sections were then weighed and trimmed to remove the portions that were not pressed between the clamps. The interior zones (accurately weighed samples that ranged from 0.95 to 1.05 g) were then crushed and homogenized in a cold room in a pre-cooled porcelain mortar with pestle after addition of 10.0 ml of cold heptane–isopropanol 3:7 that contained 50 μg/ml of cholesteryl butyrate and 12.5 μg/ml of dimyristoylglycerol as internal standards. The homogenized tissue was allowed to stand in the solvent mixture 2 h in the cold room to ensure complete lipid extraction. The extract was then filtered through a 25-mm diameter, 0.2-μm membrane filter (GA-8 Supor) in a syringe filter holder (filter 60300 and holder 4320, Gelman Sciences Inc., Ann Arbor, MI), yielding a clear and colorless extract.

Neutral lipid analysis

One-ml samples of the lipid extract were analyzed in duplicate for neutral lipids by gas–liquid chromatography (GLC). Each one ml of the extract was diluted by the further addition of 3 ml of heptane–isopropanol 3:7 (v/v) and acidified with 5 ml of 0.033 N H_2SO_4. After mixing for 30 sec the upper phase, which contained the neutral lipids, was transferred to a 3-ml conical tube. Polar lipids are not quantitatively removed in this solvent partition process. After evaporation of the solvent under nitrogen, the residue was redissolved in 100 μl of n-hexane. Two-μl aliquots were injected into the gas–liquid chromatograph. The GLC analyses were performed using a Varian 3700 gas chromatograph equipped with a series 8000 autosampler and a Spectra Physics SP4270 integrator. The separation was performed using 3% OV-1 on 100/120 Supelcoport (Supelco, Inc.) packed in a 50-cm glass column with inner diameter of 2 mm. Initially, the column temperature was 180°C and the temperature was increased at a rate of 15°C per min. When the temperature reached 349°C the column was held at this temperature for 7 min. This completed one cycle of the injection. Nitrogen was used as carrier gas at a flow rate of 25 $ml \cdot min^{-1}$. A flame ionization detector was utilized for analyses of the effluents. The gas chromatograph was programmed to print out μg in each peak, retention time, area in each peak, and retention time relative to cholesteryl butyrate. This GLC analysis provided the analytical measurement of free cholesterol and molecular species of cholesteryl esters and triacylglycerols (25, 26). However, as revealed from the GLC chromatogram (not shown), the analysis of diacylglycerols was interfered with by the presence of other lipids in the liver extracts that had retention times similar to those of diacylglycerols. It is possible that these other lipids were free diacylglycerols that became partially dehydrated and/or pyrolysis products of residual glycerophospholipids. Therefore, the diacylglycerols were purified by thin-layer chromatography prior to the GLC analysis, as described below.

Diacylglycerol analysis

One-ml samples of the lipid extract in duplicate were placed in centrifuge tubes, evaporated to dryness under nitrogen, dissolved in 40 μl of chloroform (which contained 1% ethanol), and applied to thin-layer chromatography (TLC) plates of silica gel 60-G (E. Merck, distributed by VWR Scientific, Houston, TX, cat. no. EM-7731-3). It was not necessary to quantitatively transfer the lipids in

the centrifuge tubes to the TLC plates, owing to the presence of the dimyristoylglycerol internal standard. The plates were developed with a solvent system of 75 ml hexane, 25 ml diethyl ether, and 1 ml of glacial acetic acid. Recovery of the dimyristoylglycerol internal standard determined by GLC was found to be approximately 70%. A standard containing trioleoylglycerol, oleic acid, and dioleoylglycerol was applied to adjacent lanes. After migration was complete, the plates were dried in air for 1 h and 45 min. The lanes containing the liver lipids were covered and the standard lanes were stained with a light stream of iodine vapor. The diacylglycerol zones were clearly separated and these zones were scraped into 4 ml of heptane–isopropanol 3:7 which contained 12.5 μg of cholesterol butyrate. Since diacylglycerol and free cholesterol migrate with similar R_f values in the TLC system used, these lipids are eluted together from the TLC adsorbent. The 1, 2- and 1, 3-diacylglycerols are only partially resolved and these were also eluted together. Each heptane–isopropanol extract was centrifuged to remove the silica gel. The supernatant was transferred to a 25-ml round-bottom flask and evaporated to dryness under vacuum in a rotatory evaporator at 25°C. The sample was redissolved in 5 ml of acetic anhydride–pyridine 1:1 (v/v), heated at 100°C in a sand bath for 10 min, and GLC analyses were performed as described above.

The separation of a standard mixture of (diglycerides) DG-32, DG-34, DG-36, DG-38, and DG-40, with cholesterol butyrate internal standard having a retention time of 6.50 min, is shown in **Fig. 1**. These standards were 1,3-dipalmitoylglycerol, 1-palmitoyl-3-stearoylglycerol, 1,2-dioleoylglycerol, 1-stearoyl-3-arachidonoylglycerol, and 1,3-di-11-eicosenoylglycerol. We have observed that diacylglycerols with saturated and unsaturated fatty acids, for example distearoylglycerol and dioleoylglycerol, do not separate when they contain the same number of carbons. Also, 1,2-dipalmitoylglycerol and 1,3-dipalmitoylglycerol are not resolved under the experimental conditions used.

Fatty acid composition of liver diacylglycerol

For the analysis of fatty acid composition, the freeze-clamped liver samples were extracted as described above without the diacylglycerol internal standard, since added dimyristoylglycerol would contribute myristic acid to the fatty acid mixture. Instead, duplicate 1-ml samples of the liver lipid extracts were each added to 1 ml of heptane – isopropanol 3:7 which contained 12.5 μg of dimyristoylglycerol for diacylglycerol molecular species analysis (above) and another pair of 1-ml samples of the liver lipid extracts was each added to 1 ml of heptane–isopropanol 3:7 which contained 12.5 μg of dipentadecanoylglycerol for analysis of the fatty acid composition. The dipentadecanoylglycerol could not be used as internal standard for the diacylglycerol molecular species analysis since this diacylglycerol (DG-30) has a retention time similar to the unknown lipid shown in Fig. 2. This unknown could not be DG-30 (14:0 + 16:0) since it has a retention time slightly higher than DG-30 (7.9 min vs 8.2 min) and there is insufficient 14:0 in liver diacylglycerols, as shown later. These extracts were then separated by thin-layer chromatography as described above. The diacylglycerol bands were scraped into 1 ml of hexane. The slurry of silica gel and hexane was evaporated to dryness with nitrogen, saponified with 4.7 ml of 95% ethanol and 0.3 ml of 33% KOH for 30 min at 60°C, cooled in an ice bath, acidified by addition of 5 ml of H_2O and 0.5 ml of 5.7 N HCl, and extracted twice with 2 ml hexane. The combined hexane extracts were evaporated to less than 1.0 ml with nitrogen. Fatty acids were methylated with 2 ml of BF_3 (14%) in methanol at 100°C for 10 min. After addition of 5 ml H_2O, the methyl esters were extracted twice with 2 ml of hexane. The extracts were concentrated under nitrogen and utilized for GLC analysis of fatty acid composition (27). Six livers from chow-fed rats and six from rats fed

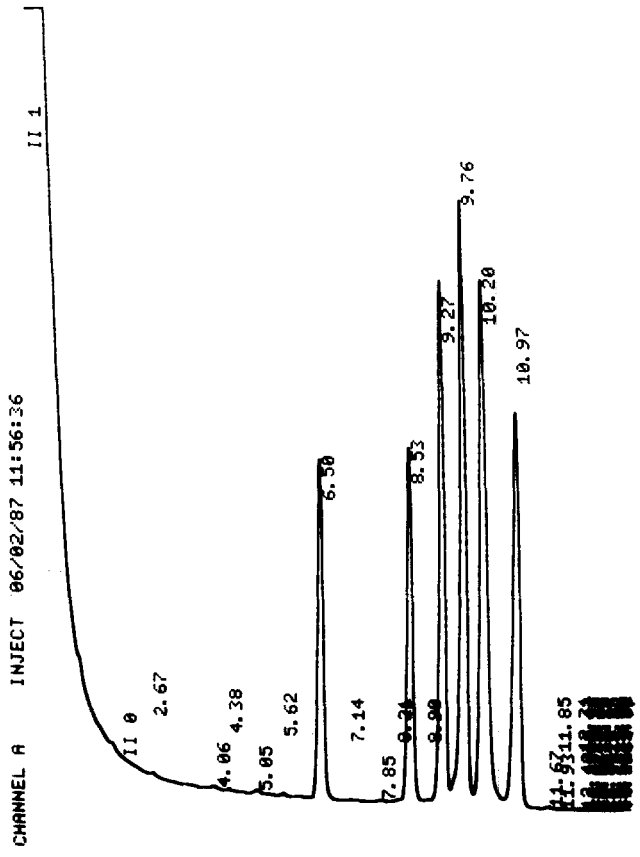

Fig. 1. Gas–liquid chromatogram of a standard mixture containing cholesteryl butyrate and diacylglycerols DG-32, DG-34, DG-36, DG-38, and DG-40. This designation of diacylglycerol molecular species indicates the number of carbons in the fatty acyl chains. The retention times of these standards were 6.50, 8.53, 9.27, 9.76, 10.20, and 10.97, respectively. The fatty acid composition of these diacylglycerol standards is described in Experimental Procedures.

the lipogenic diet for 10 days were processed for these analyses.

Serum lipids

Serum triacylglycerol and cholesterol were measured by kit assays. The triacylglycerol procedure was previously described (28). Total cholesterol was analyzed by an enzymatic cholesterol oxidase method following hydrolysis of cholesteryl esters with cholesterol esterase. In this kit assay procedure, H_2O_2 is generated and reacts with 4-aminoantipyrene and phenol to produce a pink color (#65064/93, EM Diagnostic Systems, Gibbstown, NJ, procured from Scientific Products, McGaw Park, IL).

Analysis of results

For statistical analyses Student's t test was used. Pearson correlational analyses of all measured variables were carried out using the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Results are reported for significance at the $P < 0.05$ level.

RESULTS

Body weight, liver weight, and serum lipids

Consumption of the lipogenic diet for 10 days did not significantly alter the body weights relative to those of the rats in the control group (**Table 1**). The liver weights were increased 14% ($P < 0.01$). Serum triacylglycerol and cholesterol did not appreciably differ in the two animal groups. Rats were not fasted, since effects of the lipogenic diet on liver lipids were the major focus of the present study. Chylomicrons accordingly contributed to some extent to the serum triacylglycerol in the chow-fed rats, since this diet contained 4% fat.

Neutral lipid analyses of liver extracts

In this study we utilized the freeze-clamping technique for obtaining liver tissue; therefore, the resulting analytical data of liver lipids can be anticipated to be very close to the concentration in vivo since hepatic metabolism was instantaneously stopped by lowering the temperature to that of liquid nitrogen. Because of the presence of interfering material in the liver extracts, we were unable to analyze diacylglycerols by GLC directly. It was necessary to include a purification step of TLC for the analysis of diacylglycerols. In contrast, the analysis of free cholesterol, cholesteryl esters, and triacylglycerols does not require purification prior to GLC analysis. With the presence of the dimyristoylglycerol internal standard, a reliable analysis of diacylglycerol mass of liver extracts was possible based on the degree of recovery of dimyristoylglycerol. Molecular species of diacylglycerol that contained fatty acid acyl chains with a total of 32, 34, 36, 38, and 40 carbons were resolved in this procedure.

A representative GLC tracing of the acetylated lipids in the diacylglycerol band from TLC is shown in **Fig. 2.** The large peak at 5.73 min is cholesterol acetate. The added standard cholesteryl butyrate and internal standard dimyristoylglycerol appear at 6.58 and 7.26 min, respectively. The peak at 8.20 is an unknown present in all extracts but does not interfere. DG-32, DG-34, DG-36, DG-38, and DG-40 are well separated and have retention times of 8.53, 9.16, 9.73, 10.20, and 10.74, respectively.

Effect of lipogenic diet on liver lipids

An approximately twofold increase in liver triacylglycerol content was observed in the rats fed the lipogenic diet. Liver diacylglycerols were elevated fourfold. There were also elevations in the liver free cholesterol (30%) and cholesteryl ester (51%) concentrations, although these in-

TABLE 1. Effects of a high-carbohydrate fat-free diet on body weight, liver weight, and liver lipids

	Control (12)[a]	Lipogenic Diet (12)	P value
Initial body weight, g	276 ± 15[b]	278 ± 16	NS[c]
Final body weight, g	324 ± 18	328 ± 25	NS
Liver weight, g	12.8 ± 1.0	14.6 ± 1.8	< 0.01
Serum triacylglycerol, mg/dl	98.4 ± 19.9	98.9 ± 18.2	NS
Serum total cholesterol, mg/dl	45.3 ± 9.7	46.5 ± 18.2	NS
Liver triacylglycerol, μg/g[d]	3964 ± 1401	8482 ± 3652	< 0.001
Liver diacylglycerol, μg/g	102 ± 27	392 ± 155	< 0.001
Liver free cholesterol, μg/g	1622 ± 130	2103 ± 382	< 0.001
Liver esterified cholesterol, μg/g	374 ± 82	565 ± 174	< 0.005
Liver total cholesterol, μg/g	1996 ± 182	2668 ± 342	< 0.001

[a]The number of animals in each group is shown in parentheses.
[b]Standard deviations.
[c]NS is not significant ($P > 0.05$).
[d]Values are μg/g liver wet weight.

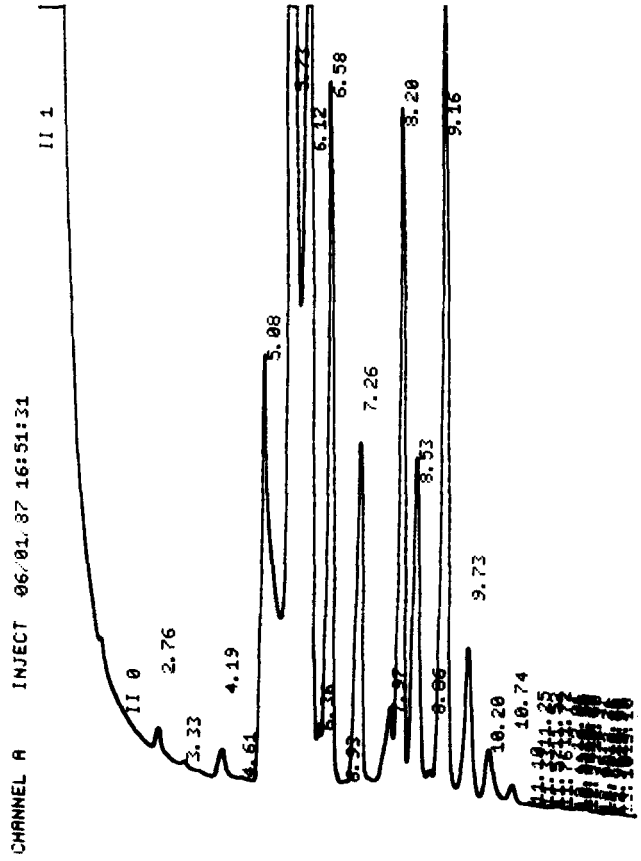

Fig. 2. Gas-liquid chromatogram of rat liver lipids eluted from the diacylglycerol band of TLC and acetylated. Cholesteryl butyrate, standard DG-28 (dimyristoylglycerol) and liver cholesterol (present as cholesteryl acetate), DG-32, DG-34, DG-36, DG-38, and DG-40 appear at 6.58, 7.26, 5.73, 8.53, 9.16, 9.73, 10.20, and 10.74 min, respectively.

creases were much less than found in liver acylglycerols.

Correlation analyses indicated several interesting relationships. Comparison of liver triacylglycerol and cholesterol contents indicated that in both control and lipogenic diet groups there was a high degee of correlation between the triacylglycerol and cholesteryl ester concentrations (**Fig. 3**). It was also found that in the lipogenic diet group the liver triacylglycerol concentration was significantly correlated with liver weight ($r = 0.62$, $P < 0.05$). No such correlation was observed in the control group. Since liver weight was correlated with body weight, the liver triacylglycerol concentration in the rats fed the lipogenic diet was found to be also correlated with body weight ($r = 0.69$, $P < 0.05$). As anticipated, the correlation was due to the weight gain in the feeding period ($r = 0.71$, $P < 0.01$).

Effect of lipogenic diet on acylglycerol and cholesteryl ester molecular species of liver

As shown in Table 1, feeding a lipogenic diet to rats leads to accumulation of liver diacylglycerols. The extent of increase in diacylglycerols was found to be related to

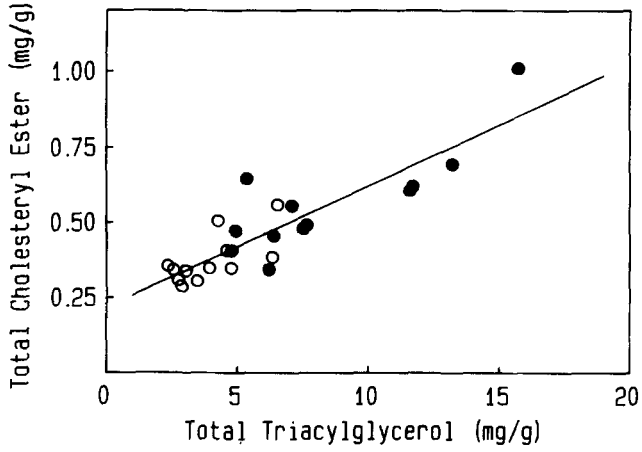

Fig. 3. Liver total triacylglycerols versus total cholesteryl esters. Values are plotted in mg/g wet wt of liver. Open circles are the 12 livers from chow-fed rats and closed circles are those from rats fed the lipogenic diet. The correlation was highly significant with $r = 0.873$ and $P < 0.001$. The values in the control group alone were $r = 0.676$ and $P < 0.05$, and those in the lipogenic diet group were $r = 0.821$ and $P < 0.005$.

the acyl-carbon numbers (**Table 2**). There was about a sevenfold increase in DG-32, a fourfold increase in DG-34, a threefold increase in DG-36, and a twofold increase in DG-38 and no change in DG-40. Apparently, the degree of the increase was reduced with each increase in acyl-chain length.

In the analyses of liver triacylglycerols (Table 2), it was found that, despite the increase of total triacylglycerol concentration in the lipogenic diet group, there was ac-

TABLE 2. Effects of a high-carbohydrate fat-free diet on molecular species of rat liver diacylglycerols, triacylglycerols, and cholesteryl esters

	Control (12)[a]	Lipogenic Diet (12)	*P* Value
	μg/g[b]		
Diacylglycerols			
32[c]	8 ± 2[d]	75 ± 31	< 0.001
34	54 ± 15	219 ± 101	< 0.001
36	20 ± 7	63 ± 26	< 0.001
38	13 ± 4	27 ± 14	< 0.005
40	7 ± 3	8 ± 5	NS[e]
Triacylglycerols			
48	88 ± 40	1099 ± 502	< 0.001
50	462 ± 199	3058 ± 1302	< 0.001
52	2022 ± 664	3516 ± 1549	< 0.01
54	830 ± 290	789 ± 373	NS
56	569 ± 322	30 ± 21	< 0.001
Cholesteryl esters			
16	187 ± 53	377 ± 87	< 0.001
18	330 ± 76	502 ± 192	< 0.01
20	112 ± 25	35 ± 25	< 0.01

[a]The number of animals in each group is shown in parentheses.
[b]Values are μg/g liver wet weight.
[c]Indicates the number of fatty acyl carbons.
[d]Standard deviations are given.
[e]NS is not significant ($P > 0.05$).

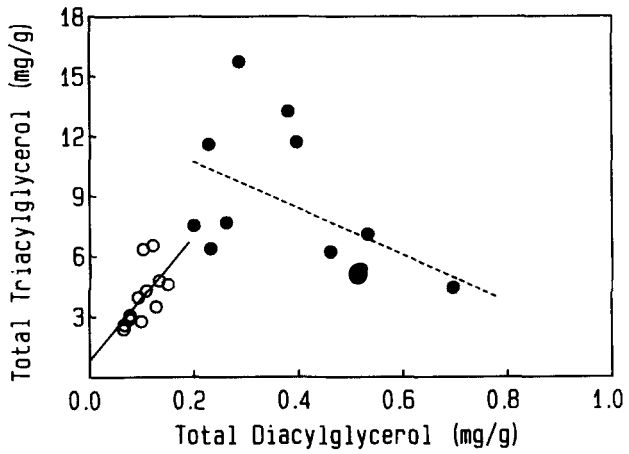

Fig. 4. Liver total triacylglycerols versus total diacylglycerols. Values are plotted in mg/g wet wt of liver. Open circles are the values from chow-fed rats and closed circles are those from rats fed the lipogenic diet. A significant correlation was observed in the chow-fed rats with $r = 0.62$ and $P < 0.05$. At the high concentrations of liver diacylglycerols present in the lipogenic diet group, liver diacylglycerols and triacylglycerols did not increase in proportion.

tually no change in the TG-54 concentration and the TG-56 content decreased. With the control and lipogenic diets, TG-52 represents the major molecular species of triacylglycerols of the liver. With the feeding of a high carbohydrate diet, there was a 12-fold increase of TG-48, a 7-fold increase of TG-50, and a 1.7-fold increase of TG-52.

A similar trend in the effects of the lipogenic diet on cholesteryl ester molecular species was found (Table 2). There was approximately a 100% increase in CE-16, a 50% increase in CE-18, and 70% decrease in CE-20 upon feeding the lipogenic diet.

Diacylglycerol versus triacylglycerol contents

As the diacylglycerol concentration in the liver increased in the control group, the triacylglycerol content was also increased ($r = 0.62$, $P < 0.05$; **Fig. 4**). At the high concentrations of diacylglycerol present in the lipogenic diet group, the liver diacylglycerol and triacylglycerol contents were not correlated (Fig 4). The diacylglycerol concentrations in the chow and lipogenic diet groups ranged from 66 to 150 μg/g wet wt and from 200 to 695 μg/g wet wt, respectively.

Fatty acid composition of diacylglycerols

Since our analyses of diacylglycerols required initial purification by thin-layer chromatography, we also performed fatty acid analyses to examine the possible random or nonrandom distribution of the fatty acyl chain in forming the various molecular species of diacylglycerols. As shown in **Table 3,** the fatty acid compositions of liver diacylglycerols in the two animal groups are quite different. The feeding of the lipogenic diet led to increases in the molar percent compositions of 14:0, 16:0, 16:1, and 18:1, and decreases in 18:0, 18:2, and 20:4. As expected, the change in composition of the fatty acids available for the synthesis of diacylglycerols led to changes in the relative abundance of molecular species of diacylglycerols. However, based on the observed fatty acid composition of diacylglycerols (Table 3) and on the diacylglycerol molecular species present in these livers, fatty acids were not distributed randomly in the diacylglycerols **(Table 4).** Specifically, the DG-34 was significantly higher and the DG-36 was significantly lower than anticipated, based on a purely random distribution of fatty acids in both control and lipogenic diet groups (Table 4). Also, DG-38 in the lipogenic diet group and DG-40 in both groups were higher than predicted from random distribution. In terms of the observed percent molar composition, the DG-34 was not altered (57.13 vs 55.12, Table 4). The decreases in the observed (experimental) percentages of DG-36, DG-38, and DG-40 in the lipogenic diet group were compensated by the increase in DG-32.

DISCUSSION

Diacylglycerols are major intermediates in the synthesis of triacylglycerols and phospholipids. They also are messengers in hormone signal transduction. It is therefore desirable to be able to quantitatively measure the spectrum of diacylglycerols present in tissues. In this study we have utilized combined TLC and GLC to analyze the individual molecular species and total mass of diacylglycerol in the liver in vivo. The total diacylglycerol content of liver in control rats averaged 102 μg/g Table 1), equivalent to about 180 μM. The range of values observed (66–150 μg/g wet wt) was within the range reported to be present in hepatocytes by Preiss et al. (29) with a diacylglycerol kinase method and by Bocckino et al. (30), who employed a TLC-Coomassie Blue procedure. The distinct advantage of the method reported herein is that molecular species are also quantified.

TABLE 3. Effect of a lipogenic diet on fatty acid composition of rat liver diacylglycerols

Fatty Acid	Control (6)[a]	Lipogenic Diet (6)	*P* Value
	mol %		
14:0	1.20 ± 0.68[b]	6.06 ± 4.41	< 0.05
16:0	26.49 ± 5.29	33.49 ± 1.69	< 0.05
16:1	3.07 ± 2.36	8.77 ± 1.32	< 0.001
18:0	16.30 ± 5.94	6.82 ± 2.66	< 0.01
18:1	23.03 ± 6.39	38.90 ± 3.98	< 0.001
18:2	18.95 ± 3.30	2.74 ± 1.03	< 0.001
20:4	10.95 ± 6.55	3.23 ± 2.21	< 0.05

[a]The number of animals in each group is shown in parentheses.
[b]Mean ± SD.

TABLE 4. Comparison of calculated and experimentally determined diacylglycerol composition in rat liver

	Control (6)[a]		Lipogenic Diet (6)	
	Experimental	Calculated[b]	Experimental	Calculated
	mol %		*mol %*	
DG-32	7.40 ± 1.84	10.65 ± 4.94	21.24 ± 3.04[c]	24.94 ± 3.59
DG-34	57.13 ± 2.92	34.64 ± 7.37[d]	55.12 ± 1.71	43.74 ± 3.40[d]
DG-36	19.34 ± 3.19	40.24 ± 5.09[d]	15.63 ± 1.98[e]	27.86 ± 3.78[d]
DG-38	10.85 ± 1.11	12.89 ± 7.77	6.41 ± 1.21[c]	3.30 ± 2.32[f]
DG-40	5.27 ± 1.24	1.57 ± 1.70[g]	1.61 ± 0.61[c]	0.16 ± 0.18[d]

[a]The number of animals in each group is shown in parentheses. All data are mean ± SD.
[b]Calculated from the data in Table 3 and based on the random distribution of the two acyl chains in the diacylglycerols. The total liver diacylglycerol in these animals increased from 88 ± 18 μg/g liver wet wt in the control group to 269 ± 70 μg/g liver wet wt in the lipogenic diet group ($P < 0.001$).
[c]$P < 0.001$ between control and lipogenic diet.
[d]$P < 0.001$ between experimental and calculated.
[e]$P < 0.05$ between control and lipogenic diet.
[f]$P < 0.05$ between experimental and calculated.
[g]$P < 0.005$ between experimental and calculated.

The correlation of liver diacylglycerol and triacylglycerol concentration in the control group (Fig. 4) suggests that the diacylglycerol concentration may be a rate-limiting factor in the synthesis of triacylglycerols. In contrast, in the lipogenic diet group, there was no positive correlation between liver diacylglycerol and triacylglycerol and, in fact, the calculated slope was negative (Fig. 4). This supports the view that under this nutritional condition, in which the diacylglycerols are more rapidly produced, diacylglycerols achieve such high concentrations that they are no longer rate-limiting in triacylglycerol synthesis. In this situation diacylglycerol acyltransferase activity (14) may be rate-limiting. Further information is required, however, to enable a conclusion on the subject. Specific sites of diacylglycerol formation include phosphatidic acid phosphatase in endoplasmic reticulum (14), phospholipase C in plasma membranes (15–18, 29, 30), and triacylglycerol lipase in lysosomes (31). Diacylglycerol concentrations at intracellular sites of formation and utilization are not known.

Enhanced hepatic synthesis of triacylglycerol in the rats fed the lipogenic diet clearly exceeded the capacity of the liver to secrete triacylglycerol in verly low density lipoproteins, as evidenced by the accumulation of liver triacylglycerol in these animals (Table 1). This increase in liver triacylglycerol content is probably a consequence of expansion of all liver triacylglycerol pools, namely lipid droplets, triglyceride-rich lipoprotein species in the secretory pathway, and the membrane-bound pool of newly synthesized triacylglycerol (28, 32). Despite this increased hepatic synthesis of triglyceride well established to occur in animals fed the fat-free sucrose-rich diet (1–13), serum triacylglycerols were not elevated and this may be a result of enhanced serum triacylglycerol removal, owing to increased insulin secretion and lipoprotein lipase activity (33, 34).

The significant correlation of body weight-gain and liver triacylglycerol concentration in rats fed the lipogenic diet (see Results) indicated that the rate of triacylglycerol synthesis in the liver is closely coupled to the body weight-gain process. The weight gained by the animals varied considerably and this was undoubtedly a reflection of differences in caloric intake. Since the lipogenic diet was fat-free, liver triacylglycerol was largely derived from de novo fatty acid synthesis. The rate of this synthetic process is dependent on the quantity of carbohydrate supplied via the portal circulation, especially when plasma free fatty acids are low and insulin is high, as in the rats fed the lipogenic diet. Triacylglycerol synthesis and storage in the liver was accordingly proportional to influx of carbohydrate.

Apparently there is a coordinated synthesis of triacylglycerol and cholesteryl ester (Fig. 3). Much of the triglyceride is accumulated in lipid droplets (28, 32). Cholesteryl esters may be sequestered in these hydrophobic structures. Alternatively, the coordinated increase in triacylglycerol and cholesteryl ester may result from the sharing of the common metabolite acyl-CoA in the final step of formation of cholesteryl ester and triacylglycerol. However, it is also possible that there is concerted regulation of cholesteryl ester synthetase and diacylglycerol acyl transferase. The differentiation of these, as well as other possibilities, will require additional future studies.

In this study we have also examined the fatty acid composition of diacylglycerols. It is apparent that the diacylglycerol composition is also directly related to the diet regimen. From the increase in total triacylglycerol (Table 1) and the fatty acid composition of these lipids (Table 3),

it was calculated that the lipogenic diet increased the 14:0, 16:0, 16:1, 18:0, and 18:1 present in liver diacylglycerols approximately 20-, 5-, 12-, 1.5-, and 7-fold, respectively. The net mass increases in these fatty acids were 23, 104, 31, 10, and 129 μg/g liver, respectively. The increaes in 14:0, 16:0, and 18:0 may be attributed to increased de novo fatty acid synthesis from carbohydrate. The increases in 16:1 and 18:1 may be due to the combined effect of fatty acid synthesis and the increase of fatty acyl-CoA desaturase activity with the lipogenic diet (35). Based on the diacylglycerol molecular species distribution (Table 3) and fatty acid composition (Table 4), it is clear that the distribution of the two fatty acids on the diacylglycerol molecule cannot be described by random distribution. For this reason, we conclude that the formation or degradation of diacylglycerol must not be a random event with respect to the fatty acyl-chain of the diacylglycerols. Thus, the enzyme-catalyzed reactions involved with the formation and/or degradation of diacylglycerol must also exhibit acyl-chain and/or positional specificity. The present results, indicative of nonrandom distribution of fatty acids in liver diacylglycerols, are consistent with earlier reports of a nonrandom distribution of fatty acids in liver triacylglycerols (36, 37).

The increased concentration of liver diacylglycerols in rats fed the lipogenic diet could arise from more active biosynthesis of diacylglycerols in the process of triacylglycerol formation, or from increased lipolytic degradation of the expanded triacylglycerol pool, or from both processes. Increased phospholipase activity is not included here, since DG-32 and DG-34 were most affected (Table 2). In view of the enhanced state of hepatic triacylglycerol synthesis and secretion in these animals (1–13), elevated diacylglycerol formation via phosphatidic acid phosphatase in the endoplasmic reticulum (14) is probably the major contributor. Further, since triacylglycerol-rich lipoproteins are secreted, the synthesis of hepatic triacylglycerol clearly exceeds its degradation in the steady state when the diet is fat-free. This also favors the concept that increased formation of diacylglycerol from phosphatidic acid is the most significant source of the elevated liver diacylglycerol in the lipogenic diet group. This remains tentative, since the kinetic properties of the enzymes which influence the pool sizes of diacylglycerols in triacylglycerol synthesis and degradation are not yet defined.

In conclusion, in this study we have developed a method for the analysis of rat liver diacylglycerol by GLC. We have applied this procedure in determining the effect of a fat-free, high-carbohydrate lipogenic diet on the rat liver neutral lipid profile. The results clearly demonstrate the effect of this diet in causing the increase of steady state concentration of diacylglycerols and other neutral lipids.

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REFERENCES

1. Hill, R., J. M. Linazasoro, F. Chevalier, and I. L. Chaikoff. 1958. Regulation of hepatic lipogenesis: influence of dietary fats. *J. Biol. Chem.* **233:** 305–310.
2. Fritz, I. B. 1961. Factors influencing the rates of long chain fatty acid oxidation and synthesis in mammalian systems. *Physiol. Rev.* **41:** 52–129.
3. Tepperman, J., and H. M. Tepperman. 1970. Gluconeogenesis, lipogenesis and the Sherringtonian metaphor. *Fed. Proc.* **29:** 1284–1293.
4. Porter, J. W., S. Kuman, and R. E. Dugan. 1971. Synthesis of fatty acids by enzymes of avian and mammalian species. *Prog. Biochem. Pharmacol.* **6:** 1–101.
5. Brunengraber, H., M. Boutry, and J. M. Lowenstein. 1973. Fatty acid and 3-β-hydroxysterol synthesis in the perfused rat liver. *J. Biol. Chem.* **248:** 2656–2669.
6. Gibson, D. M., R. T. Lyons, D. F. Scott, and Y. Muto. 1972. Synthesis and degradation of the lipogenic enzymes of rat liver. *Adv. Enzyme Regul.* **10:** 187–204.
7. Numa, S., and S. Yamashita. 1974. Regulation of lipogenesis in animal tissues. *Curr. Top. Cell Regul.* **8:** 197–246.
8. Van Golde, L. M. G., and S. G. Van den Bergh. 1977. Liver. *In* Lipid Metabolism in Mammals. F. Snyder, editor. Plenum Press, New York. Vol. 1: 35–149.
9. Groener, J. E. M., and L. M. G. van Golde. 1977. Effect of fasting and feeding a high sucrose, fat-free diet on the synthesis of hepatic glycerolipids in vivo and in isolated hepatocytes. *Biochim. Biophys. Acta.* **487:** 105–114.
10. Bird, M. I., and M. A. Williams. 1982. Triacylglycerol secretion in rats: effects of essential fatty acids and influence of dietary sucrose, glucose or fructose. *J. Nutr.* **112:** 2267–2278.
11. Boogaerts, J. R., M. Malone-McNeal, J. Archambault-Schexnayder, and R. A. Davis. 1984. Dietary carbohydrate induces lipogenesis and very low density lipoprotein synthesis. *Am. J. Physiol.* **246:** E77–E83.
12. Windmueller, H. G. and A. E. Spaeth. 1985. Regulated biosynthesis and divergent metabolism of three forms of hepatic apolipoprotein B in the rat. *J. Lipid Res.* **26:** 70–81.
13. Yamamoto, M., I. Yamamoto, Y. Tanaka, and J. A. Ontko. 1987. Fatty acid metabolism and lipid secretion by perfused livers from rats fed laboratory stock and sucrose-rich diets. *J. Lipid Res.* **28:** 1156–1165.
14. Bell, R. M., L. M. Ballas, and R. A. Coleman. 1981. Lipid topogenesis. *J. Lipid Res.* **22:** 391–403.
15. Berridge, M. J. 1984. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* **220:** 345–360.
16. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature.* **308:** 693–698.
17. Exton, J. 1986. Mechanisms involved in calcium-mobilizing agonist responses. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **20:** 211–262.

18. Abdel-Latif, A. A. 1986. Calcium-mobilizing receptors, phosphoinositides, and the generation of second messengers. *Pharmacol. Rev.* **38:** 227–272.
19. Hohorst, H. J., F. H. Kreutz, and T. Bucher. 1959. Über metabolitgehalt und metabolit-konzentrationen in der leber der ratte. *Biochem. Z.* **332:** 18–46.
20. Wollenberger, A., O. Ristau, and G. Schoffa. 1960. Eine einfache technik der extrem schnellen abkühlung größerer gewebestücke. *Pflügers Arch. Physiol.* **270:** 399–412.
21. Williamson, J. R., and B. E. Corkey. 1969. Assays of intermediates of the citric acid cycle and related compounds by fluorometric enzyme methods. *Methods Enzymol.* **13:** 434–513.
22. Ontko, J. A., and P. J. Randle. 1967. Inhibition of glucose utilization by perfusion with chylomicrons in rat heart. *Biochem. J.* **104:** 43C–44C.
23. Yamamoto, M., N. Fukuda, J. Triscari, A. C. Sullivan, and J. A. Ontko. 1985. Decreased hepatic production of very low density lipoproteins following activation of fatty acid oxidation by Ro 22-0654. *J. Lipid Res.* **26:** 1196–1204.
24. Azain, M. J., N. Fukuda, F-F. Chao, M. Yamamoto, and J. A. Ontko. 1985. Contributions of fatty acid and sterol synthesis to triglyceride and cholesterol secretion by the perfused rat liver in genetic hyperlipemia and obesity. *J. Biol. Chem.* **260:** 174–181.
25. Kuksis, A., J. J. Myher, L. Marai, and K. Geher. 1975. Determination of plasma lipid profiles by automated gas chromatography and computerized data analysis. *J. Chromatogr. Sci.* **13**: 423–430.
26. Wang, C-S., N. Fukuda, and J. A. Ontko. 1984. Studies on the mechanism of hypertriglyceridemia in the genetically obese Zucker rat. *J. Lipid Res.* **25:** 571–579.
27. Dashti, N., and J. A. Ontko. 1983. Alterations in rat serum lipids and apolipoproteins following clofibrate treatment. *Atherosclerosis.* **49:** 255–266.
28. Ontko, J. A., L. W. Perrin, and L. S. Horne. 1986. Isolation of hepatocellular lipid droplets: the separation of distinct subpopulations. *J. Lipid Res.* **27:** 1097–1103.
29. Preiss, J., C. R. Loomis, W. R. Bishop, R. Stein, J. E. Niedel, and R. M. Bell. 1986. Quantitative measurement of *sn*-1,2-diacylglycerols present in platelets, hepatocytes and *ras*- and *sis*-transformed normal rat kidney cells. *J. Biol. Chem.* **261:** 8597–8600.
30. Bocckino, S. B., P. F. Blackmore, P. B. Wilson, and J. H. Exton. 1987. Phosphatidate accumulation in hormone-treated hepatocytes via a phospholipase D mechanism. *J. Biol. Chem.* **262:** 15309–15315.
31. Burrier, R. E., and P. Brecher. 1983. Hydrolysis of triolein in phospholipid vesicles and microemulsions by a purified rat liver acid lipase. *J. Biol. Chem.* **258:** 12043–12050.
32. Chao, F-F., D. L. Stiers, and J. A. Ontko. 1986. Hepatocellular triglyceride synthesis and transfer to lipid droplets and nascent very low density lipoproteins. *J. Lipid Res.* **27:** 1174–1181.
33. Nilsson-Ehle, P., A. S. Garfinkel, and M. C. Schotz. 1980. Lipolytic enzymes and plasma lipoprotein metabolism. *Annu. Rev. Biochem.* **49:** 667–693.
34. Hamosh, M., and P. Hamosh. 1983. Lipoprotein lipase: its physiological and clinical significance. *Mol. Aspects Med.* **6:** 199–289.
35. Jeffcoat, R. 1979. The biosynthesis of unsaturated fatty acids and its control in mammalian liver. *Essays Biochem.* **15:** 1–36.
36. Blank, M. L., B. Verdino, and O. S. Privett. 1965. Determination of triglyceride structure via silver nitrate-TLC. *J. Am. Oil Chem. Soc.* **42:** 87–90.
37. Slakey, P. M., and W. E. M. Lands. 1968. The structure of rat liver triglycerides. *Lipids.* **3:** 30–36.