

## Reciprocal responses to dietary diacylglycerol of hepatic enzymes of fatty acid synthesis and oxidation in the rat

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The activities of hepatic enzymes of fatty acid synthesis and oxidation were compared in rats fed on diacylglycerol and triacylglycerol. In the first trial, rats were fed on diacylglycerol or triacylglycerol (rapeseed oil) for 14 d. The diacylglycerol preparation contained 65.2 g and 32.6 g fatty acids/100 g total fatty acids as 1,3-species and 1,2-species respectively. Fatty acid compositions of these dietary lipids were similar. Dietary acylglycerols were added to experimental diets to provide the same amounts of fatty acids (93.9 g/kg diet). Dietary diacylglycerol compared with triacylglycerol significantly reduced the concentrations of serum and liver triacylglycerol. The activities of enzymes of fatty acid synthesis (fatty acid synthetase, glucose 6-phosphate dehydrogenase (*EC* 1.1.1.49) and malic enzyme (*EC* 1.1.1.40)) were significantly lower in rats fed on diacylglycerol than in those fed on triacylglycerol. In contrast, the rates of mitochondrial and peroxisomal oxidation of palmitoyl-CoA in liver homogenates were higher in rats fed on diacylglycerol than in those fed on triacylglycerol. In the second trial, varying amounts of dietary triacylglycerol were replaced by diacylglycerol while the dietary fatty acid content was maintained (93.9 g/kg diet). After 21 d of the feeding period the significant reductions in serum and liver triacylglycerol levels were confirmed in groups of rats fed on the diets in which diacylglycerol supplied more than 65.8 g fatty acids/kg diet (65.8 and 93.9 g/kg). Reductions in the activities of enzymes of fatty acid synthesis and increases in palmitoyl-CoA oxidation rates by both mitochondrial and peroxisomal pathways were also apparent when diacylglycerol replaced triacylglycerol in diets to supply more than 65.8 g fatty acid/kg. Increasing dietary levels of diacylglycerol also progressively increased the activities of enzymes involved in the  $\beta$ -oxidation pathway (carnitine palmitoyltransferase (*EC* 2.3.1.21), acyl-CoA dehydrogenase (*EC* 1.3.99.3), acyl-CoA oxidase (*EC* 1.3.3.6), enoyl-CoA hydratase (*EC* 4.2.1.17), 3-hydroxyacyl-CoA dehydrogenase (*EC* 1.1.1.35), 2,4-dienoyl-CoA reductase (*EC* 1.3.1.34) and  $\Delta^3, \Delta^2$ -enoyl-CoA isomerase (*EC* 5.3.3.8)) in the liver. These results suggest that alteration of fatty acid metabolism in the liver is a factor responsible for the serum triacylglycerol-lowering effect of dietary diacylglycerol.

**Diacylglycerol: Fatty acid synthesis: Fatty acid oxidation: Serum triacylglycerol**

In an initial step of the digestion and absorption of dietary triacylglycerol, lingual or pancreatic lipase (*EC* 3.1.1.3) cleaves the fatty acid at the 1 or 3 position of the lipid molecule to form diacylglycerol. As diacylglycerols which occur during the digestive process of triacylglycerol are 1,2 and 2,3-species but not 1,3-species, it is expected that ingestion of 1,3-diacylglycerol compared with triacylglycerol may exert a different effect on lipid metabolism in the organism. In fact, we previously demonstrated that dietary

\*For reprints.

diacylglycerol mainly composed of 1,3-species compared with triacylglycerol (rapeseed oil) significantly reduced serum triacylglycerol concentration in rats (Hara *et al.* 1993). We also showed that intragastric infusion of diacylglycerol compared with triacylglycerol significantly retarded the lymphatic transport of triacylglycerol as chylomicrons in rats (Murata *et al.* 1994). The impairment of chylomicron assembly and subsequent release into the circulation through the lymph is therefore considered to be a factor responsible for the serum triacylglycerol-lowering effect of dietary diacylglycerol. A plausible alternative factor modifying the serum triacylglycerol concentration is the change in the rates of fatty acid synthesis and oxidation in the liver. It has been clearly demonstrated that the alterations in the rates of fatty acid synthesis (Windmueller & Spaeth, 1967; Brunengraber *et al.* 1973) and oxidation, either in the mitochondrial (Ide & Ontko, 1981) or the peroxisomal pathway (Ide *et al.* 1982), modify the availability of fatty acids for triacylglycerol synthesis and in turn alter the VLDL production by the liver. We hypothesized that dietary diacylglycerol not only alters chylomicron production but also may modify the hepatic metabolism of fatty acid and thus lower serum triacylglycerol concentration in the rat. As the effect of dietary diacylglycerol on the metabolism of fatty acid in the liver is not known, we compared the effects of dietary diacylglycerol and triacylglycerol on the activities of hepatic enzymes in fatty acid synthesis and oxidation pathways in the present study.

## EXPERIMENTAL

### *Materials*

The diacylglycerol preparation was synthesized enzymically by esterifying glycerol with fatty acids from rapeseed oil using the reverse reaction of 1,3-specific lipase, purified using silicic acid chromatography (Barron & Hanahan, 1958; Hara *et al.* 1993; Murata *et al.* 1994) and analysed (Karlson & Pascher, 1971; Hara *et al.* 1993; Murata *et al.* 1994) as described previously. The majority of fatty acids in rapeseed oil exist as triacylglycerol while fatty acids in the diacylglycerol preparation were recovered mainly in 1,3- (65.2 g/100 g total fatty acids in the preparation) and, to a lesser extent, in 1,2-diacylglycerol (32.6 g/100 g total fatty acid in the preparation) fractions (Table 1). We synthesized diacylglycerol using 1,3-specific lipase, therefore 1,2-diacylglycerol was considered to be the product of acyl migration. The fatty acid composition of the diacylglycerol preparation was indistinguishable from that of rapeseed oil. The fatty acid content of diacylglycerol was 891 g/kg and that of rapeseed oil was 939 g/kg. [ $^{14}\text{C}$ ]Palmitic acid was purchased from Amersham International, Amersham, Bucks. [ $^{14}\text{C}$ ]Palmitoyl-CoA and non-radiolabelled palmitoyl-CoA were prepared according to the method of Kawaguchi *et al.* (1981). Acetyl-CoA was prepared by acylating CoA with acetic anhydride, acetoacetyl-CoA using diketene and crotonyl-CoA using crotonic anhydride. Sorboyl-CoA and *trans*-3-hexenoyl-CoA were prepared using the mixed anhydride method (Goldman & Vagelos, 1961). Malonyl-CoA was purchased from Sigma Chemical Co., St. Louis, MO, USA. Bovine serum albumin fraction V (essential-fatty-acid-free) was the product of Boehringer Mannheim, Gmbh, Germany.

### *Animals and diets*

Male Sprague-Dawley rats obtained from Charles River Japan, Kanagawa, Japan were housed individually in a room with controlled temperature (20–22°), humidity (55–65 %) and lighting (light on from 0.700 to 19.00 hours), and fed on a commercial non-purified

Table 1. *Fatty acid distributions and compositions (g/100 g total fatty acids) of dietary triacylglycerol and diacylglycerol*

	Triacylglycerol	Diacylglycerol
Fatty acid distribution		
Triacylglycerol	98.8	0.4
1,2-Diacylglycerol	0.4	32.6
1,3-Diacylglycerol	0.8	65.2
Monoacylglycerol	—	1.7
Fatty acid composition		
16:0	3.8	4.5
16:1	0.4	0.1
18:0	1.3	1.5
18:1	60.5	60.7
18:2	22.3	23.8
18:3	11.6	10.9

diet (Type NMF, Oriental Yeast Co., Tokyo, Japan). After 7 d of acclimatization to the housing conditions, rats were fed on purified experimental diets. The basal composition of the experimental diet was (g/kg): casein 200, maize starch 150, cellulose 20, mineral mixture 35, vitamin mixture 10, DL-methionine 3, choline bitartrate 2 and sucrose to 1 kg. The compositions of the mineral and vitamin mixtures were those recommended by the American Institute of Nutrition (1977). Dietary lipids were added to the diets to provide the same amount of fatty acids (93.9 g/kg diet). In the first experiment, rats were randomly divided into two groups consisting of eight animals each and fed on experimental diets containing 100 g triacylglycerol/kg (rapeseed oil) and 105 g diacylglycerol/kg for 14 d. In the second experiment, four groups of rats consisting of seven animals each were fed on diets containing varying amounts of the diacylglycerol preparation for 21 d. The diet without diacylglycerol contained 93.9 g fatty acids/kg of the diet as rapeseed oil. Varying amounts of diacylglycerol preparation were added to the experimental diet in lieu of rapeseed oil in amounts so that this dietary lipid provided 28.2, 65.7 and 93.9 g fatty acids/kg of the diet while maintaining constant dietary fatty acid levels. We followed the guidelines of our institute for care and use of laboratory animals. Average body weights of animals at initiation of the experiments were 153 (SD 11) g for Expt 1 and 118 (SD 11) g for Expt 2.

#### *Enzyme assays*

At the termination of the experimental period, rats were anaesthetized using diethyl ether and killed by bleeding from the abdominal aorta, after which livers were quickly excised. About 3 g of each liver was homogenized with 7 volumes of 0.25 M-sucrose and centrifuged at 500 g for 10 min. The supernatant fraction was recentrifuged at 9000 g for 10 min to isolate mitochondria. The mitochondrial fraction was washed twice with 0.25 M-sucrose containing 1 mM-EDTA and 3 mM-Tris-HCl (pH 7.0) and finally suspended in the same medium to give a protein concentration of 20–25 mg/ml. Glucose 6-phosphate dehydrogenase (*EC* 1.1.1.49) (Kelley & Kletzien, 1984), malic enzyme (*EC* 1.1.1.40) (Hsu & Lardy, 1969) and fatty acid synthetase (Kelley *et al.* 1986) activities were measured in the 9000 g supernatant fraction of liver homogenates (Ide *et al.* 1992). The rates of mitochondrial and peroxisomal oxidation of fatty acids were measured using the 500 g supernatant fraction of liver homogenates according to the method described by Mannaerts

*et al.* (1979). The assay mixture for mitochondrial activity contained 4 mM-ATP, 0.5 mM-L-carnitine, 0.05 mM-CoA, 2 mM-dithiothreitol, 7.2 mg/ml albumin (fatty-acid-free), 0.2 mM-[1-<sup>14</sup>C]palmitoyl-CoA (0.0074 MBq/μmol) and 0.1 ml of the 500 g supernatant fraction (1.3–1.6 mg protein) in modified Krebs–Henseleit bicarbonate buffer (pH 7.4) (Mannaerts *et al.* 1979). Assays conducted in the presence of 2 mM-KCN served as blanks. The mixture for the assay of peroxisomal activity contained 4 mM-ATP, 0.5 mM-CoA, 2 mM-NAD, 2 mM-dithiothreitol, 2 mM-KCN, 0.2 mM-[1-<sup>14</sup>C]palmitoyl-CoA (0.0074 MBq/μmol) and 0.05 ml of the 500 g supernatant fraction (0.65–0.8 mg protein) in the modified Krebs–Henseleit bicarbonate buffer (pH 7.4). The presence of albumin is obligatory to measure mitochondrial activity while it severely inhibits peroxisomal oxidation of palmitoyl-CoA (Mannaerts *et al.* 1979). We therefore did not include albumin for the assay of peroxisomal activity. Assays conducted in the absence of the enzyme source served as blanks for peroxisomal activities. The final volumes of the mixture were 2.5 ml for both the assays of mitochondrial and peroxisomal activities. The rates of mitochondrial and peroxisomal β-oxidation of palmitoyl-CoA were assayed under an atmosphere of CO<sub>2</sub>–O<sub>2</sub> (5 : 95, v/v) for 10 min and terminated by pouring the mixture into test-tubes containing 0.625 ml 3 M-perchloric acid. After letting the mixture stand for at least 15 min in an ice bath, it was centrifuged at 2500 g for 10 min and 2 ml of the supernatant fraction was assayed for radioactivity by liquid scintillation counting. The supernatant fraction obtained after centrifugation of liver homogenates at 500 g for 10 min was used for the measurements of the activities of fatty acid oxidation enzymes except for carnitine palmitoyltransferase (*EC* 2.3.1.21) and acyl-CoA dehydrogenase (*EC* 1.3.99.3). Because of the consideration that carnitine palmitoyltransferase and acyl-CoA dehydrogenase are primarily mitochondrial enzymes (Schulz, 1991) and of the fact that the assays using the 500 g supernatant fraction as the enzyme source gave extremely high blank values, these enzymes were assayed in isolated mitochondrial fraction as an enzyme source. Carnitine palmitoyltransferase I activity was determined radiochemically using freshly isolated mitochondria as an enzyme source according to the method of Bremer *et al.* (1985). Carnitine palmitoyltransferase activity was also measured spectrophotometrically in the freeze-thawed preparation of isolated mitochondria solubilized with Triton X-100 according to the method of Markwell *et al.* (1973) as described elsewhere (Ide *et al.* 1987). The value obtained presumably represents the sum of the activities of carnitine palmitoyltransferase I and II. Acyl-CoA dehydrogenase activity was measured in isolated mitochondria according to the method described by Dommes & Kunau (1976) except that phenazine methosulphate was used as a primary electron acceptor. Acyl-CoA oxidase (*EC* 1.3.3.6) activity was measured in the 500 g supernatant fraction of liver homogenates as described elsewhere (Hashimoto *et al.* 1981; Ide *et al.* 1987). Palmitoyl-CoA was used as a substrate for carnitine palmitoyltransferase, acyl-CoA dehydrogenase and acyl-CoA oxidase assays. Crotonyl-CoA was used as the substrate for enoyl-CoA hydratase (*EC* 4.2.1.17) (Osumi & Hashimoto, 1979a), acetoacetyl-CoA for 3-hydroxyacyl-CoA dehydrogenase (*EC* 1.1.1.35) (Osumi & Hashimoto, 1979b), sorboyl-CoA for 2,4-dienoyl-CoA reductase (*EC* 1.3.1.34) (Kunau & Dommes, 1978) and *trans*-3-hexenoyl-CoA for Δ<sup>3</sup>,Δ<sup>2</sup>-enoyl-CoA isomerase (*EC* 5.3.3.8) (Palosaari *et al.* 1990) in assaying the activities. Activities of marker enzymes for cell organelles including succinate dehydrogenase (*EC* 1.3.99.1) (mitochondria) (Veeger *et al.* 1969), catalase (*EC* 1.11.1.6) (peroxisomes) (Cohen *et al.* 1970), lactate dehydrogenase (*EC* 1.1.1.27) (cytosols) (Wahlefeld, 1985) and NADPH-cytochrome C reductase (microsomes) (Omura & Takesue, 1970) were determined in the 500 g supernatant fraction of liver homogenates. The rates of mitochondrial and peroxisomal oxidation of fatty acids and carnitine

palmitoyltransferase I activity were measured on the day of slaughter using non-freeze-thawed enzyme preparations. Other enzymes were assayed in the enzyme preparations stored at  $-40^{\circ}$  for up to 10 d.

### *Lipid analyses*

Liver and serum lipids were extracted and purified (Folch *et al.* 1957). Triacylglycerol, phospholipid and cholesterol contents in the extracts were determined as described previously (Hara *et al.* 1993). Triacylglycerol and phospholipid in the liver lipid extract were separated by TLC and the fatty acid compositions of these lipid molecules were determined using a gas-liquid chromatograph (Ide *et al.* 1978).

### *Statistical analyses*

Examinations of significant differences of means with a pooled estimate of variance for Expts 1 and 2 were established appropriately according to the methods of Snedecor & Cochran (1989) for the comparison of two samples (Expt 1) and for one-way classifications (Expt 2) respectively as detailed previously (Ide *et al.* 1994).

## RESULTS

Rats were fed on diets containing 93.9 g fatty acids/kg as triacylglycerol (rapeseed oil) or the diacylglycerol preparation for 14 d in Expt 1. No significant difference in body weights was observed at the termination of the experimental period (292 (SD 20) and 300 (SD 14) g for rats fed on the triacylglycerol and diacylglycerol diets respectively). Dietary diacylglycerol did not affect amounts of experimental diets consumed during the 14 d (345 (SD 62) and 334 (SD 54) g for rats fed on the triacylglycerol and diacylglycerol diets respectively). The weights of livers excised at the termination of the experimental period were the same for rats fed on the triacylglycerol diet (55.7 (SD 6.5) g/kg body weight) and the diacylglycerol diet (51.9 (SD 11.0) g/kg body weight). In the experiment where rats were fed on diets varying in diacylglycerol content for 21 d (Expt 2), body weights at the time of slaughter were indistinguishable among the groups (288–290 g). Cumulative values for the amount of diet consumed during the experimental period ranged from 443 to 452 g with no significant differences among the groups. Also, no differences in the weights of livers excised at the termination of the experimental period (49.5–53.2 g/kg body weight) were observed among the four groups.

The potency of dietary diacylglycerol in reducing the serum triacylglycerol concentration demonstrated in a previous study (Hara *et al.* 1993) was confirmed in the present study (Table 2). Thus, the serum triacylglycerol concentration was significantly lower in rats fed on the diet containing 93.9 g fatty acids/kg exclusively as diacylglycerol for 14 d than in those fed on the diet containing the same dietary level of fatty acids in the form of triacylglycerol (Expt 1). The serum triacylglycerol concentration decreased as the dietary level of diacylglycerol increased and values in the rats fed on the diets containing 65.7 and 93.8 g fatty acids in the form of diacylglycerol became significantly lower than those in the animals fed on a diet without diacylglycerol (Expt 2). However, dietary diacylglycerol was in no way effective in altering the serum concentrations of phospholipid and cholesterol in either Expt 1 or 2. Diets containing diacylglycerol as the dietary lipid were also effective in reducing the triacylglycerol concentration in the liver in both Expts 1 and 2. The replacement of triacylglycerol with diacylglycerol to supply more than 65.7 g

Table 2. Concentrations of lipid components in the serum (mg/ml) and liver (mg/g) of rats fed on diets containing different proportions of diacylglycerol and triacylglycerol\*  
(Mean values and standard deviations)

Dietary fatty acids as diacylglycerol/ triacylglycerol (g/kg diet)	n	Lipid components					
		Triacylglycerol		Phospholipid		Cholesterol	
		Mean	SD	Mean	SD	Mean	SD
<b>Expt 1</b>							
<b>Serum lipids</b>							
0/93.9	8	3.13 <sup>b</sup>	1.28	2.18 <sup>a</sup>	0.57	1.02 <sup>a</sup>	0.14
93.9/0	8	1.89 <sup>a</sup>	0.53	2.22 <sup>a</sup>	0.40	1.03 <sup>a</sup>	0.08
Pooled SD			0.96		0.49		0.11
df			14		14		14
<b>Liver lipids</b>							
0/93.9	8	44.7 <sup>b</sup>	5.3	28.8 <sup>a</sup>	2.0	2.39 <sup>a</sup>	0.61
93.9/0	8	34.0 <sup>a</sup>	6.3	28.4 <sup>a</sup>	1.9	4.85 <sup>b</sup>	0.70
Pooled SD			5.8		1.9		0.66
df			14		14		14
<b>Expt 2</b>							
<b>Serum lipids</b>							
0/93.9	7	3.71 <sup>c</sup>	0.53	2.22 <sup>a</sup>	0.58	1.04 <sup>a</sup>	0.11
28.2/65.7	7	3.60 <sup>c</sup>	0.28	2.07 <sup>a</sup>	0.21	1.04 <sup>a</sup>	0.10
65.7/28.2	7	2.86 <sup>b</sup>	0.15	2.03 <sup>a</sup>	0.05	1.00 <sup>a</sup>	0.08
93.9/0	7	2.07 <sup>a</sup>	0.11	2.01 <sup>a</sup>	0.18	1.04 <sup>a</sup>	0.11
Pooled SD			0.34		0.35		0.11
df			24		24		24
<b>Liver lipids</b>							
0/93.9	7	31.4 <sup>b</sup>	5.18	22.7 <sup>a</sup>	2.88	2.08 <sup>a</sup>	0.52
28.2/65.7	7	33.2 <sup>b</sup>	4.54	23.3 <sup>a</sup>	2.41	2.03 <sup>a</sup>	0.33
65.7/28.2	7	27.5 <sup>a</sup>	3.64	22.6 <sup>a</sup>	2.68	2.08 <sup>a</sup>	0.31
93.9/0	7	25.5 <sup>a</sup>	2.96	22.5 <sup>a</sup>	3.47	2.93 <sup>b</sup>	0.27
Pooled SD			4.17		2.89		0.37
df			24		24		24

<sup>a,b,c</sup>Mean values within a column, within each experiment, with unlike superscript letters were significantly different,  $P < 0.05$ .

\*For details of diets and procedures, see pp. 108–111.

fatty acids in the diet was required to decrease triacylglycerol concentration in the liver effectively (Expt 2). In contrast, the concentration of liver cholesterol was significantly higher in rats fed on acylglycerol exclusively in the form of diacylglycerol than in animals fed on a diet devoid of diacylglycerol, in both Expts 1 and 2.

Compared with triacylglycerol, dietary diacylglycerol reduced the activities of various enzymes of fatty acid synthesis in both Expts 1 and 2 (Table 3). Activities decreased as the dietary levels of diacylglycerol increased (Expt 2). Even a diet which supplied 28.2 g fatty acids/kg diet in the form of diacylglycerol compared with the diet without diacylglycerol was effective in significantly reducing the activity of glucose 6-phosphate dehydrogenase. The replacement of triacylglycerol by diacylglycerol to supply more than 65.7 g of fatty acid in the diet was required to reduce the activities of malic enzyme and fatty acid synthetase.

The rate of palmitoyl-CoA oxidation through the mitochondrial pathway measured using the 500 g supernatant fraction of rat liver homogenates as an enzyme source was about 25 % higher in rats fed on diacylglycerol than in those fed on triacylglycerol in Expt 1 (Table 4). The rate of peroxisomal oxidation of the palmitoyl-CoA substrate in this

Table 3. *Activities of enzymes involved in fatty acid synthesis (nmol/min per mg protein) in the livers of rats fed on diets containing different proportions of diacylglycerol and triacylglycerol\**

(Mean values and standard deviations)

Dietary fatty acids as diacylglycerol/ triacylglycerol (g/kg diet)	n	Enzyme activities					
		Glucose 6-phosphate dehydrogenase (EC 1.1.1.49)		Malic enzyme (EC 1.1.1.40)		Fatty acid synthetase	
		Mean	SD	Mean	SD	Mean	SD
<b>Expt 1</b>							
0/93.9	8	155 <sup>b</sup>	14.2	119 <sup>b</sup>	16.7	39.1 <sup>b</sup>	17.2
93.9/0	8	90.3 <sup>a</sup>	10.1	91.8 <sup>a</sup>	16.8	20.9 <sup>a</sup>	6.8
Pooled SD			12.2		16.8		13.1
df			14		14		14
<b>Expt 2</b>							
0/93.9	7	204 <sup>c</sup>	19.8	103 <sup>c</sup>	15.1	31.1 <sup>b</sup>	6.7
28.2/65.7	7	103 <sup>b</sup>	11.0	92.7 <sup>bc</sup>	22.2	33.3 <sup>b</sup>	7.9
65.7/28.2	7	83.5 <sup>a</sup>	16.0	87.0 <sup>ab</sup>	14.6	24.6 <sup>a</sup>	5.4
93.9/0	7	76.7 <sup>a</sup>	17.7	70.4 <sup>a</sup>	15.6	20.2 <sup>a</sup>	4.1
Pooled SD			16.4		17.2		6.2
df			24		24		24

<sup>a,b,c</sup>Mean values within a column, within each experiment not sharing a common superscript letter were significantly different,  $P < 0.05$ .

\*For details of diets and procedures, see pp. 108–111.

experiment was also 40 % higher in rats fed on diacylglycerol than in those fed on triacylglycerol. In Expt 2 the rates of mitochondrial and peroxisomal oxidation of palmitoyl-CoA increased as the dietary level of diacylglycerol increased. The replacement of triacylglycerol by diacylglycerol to supply more than 65.7 g fatty acids/kg of the diet was required to obtain apparent physiological activity of dietary diacylglycerol in these variables.

Table 5 shows the activities of various enzymes involved in the  $\beta$ -oxidation pathway in rats fed on the diets containing varying amounts of diacylglycerol (Expt 2). The supernatant fraction obtained after centrifugation of liver homogenates at 500 g for 10 min was used to measure the activities of fatty acid oxidation enzymes except for carnitine palmitoyltransferase and acyl-CoA dehydrogenase; these were assayed in isolated mitochondrial fraction. The amounts of mitochondrial protein recovered from the 500 g supernatant fraction were comparable among the various groups (19.2–20.5 mg/g liver). The activity of carnitine palmitoyltransferase, which regulates the rate of transport of fatty acids across the mitochondrial membrane, measured either radiochemically using intact mitochondria as an enzyme source (carnitine palmitoyltransferase I) or spectrophotometrically using the Triton-solubilized preparation of mitochondria increased as the dietary level of diacylglycerol increased. Dietary diacylglycerol also increased the activities of various enzymes involved in the fatty acid  $\beta$ -oxidation cycle (acyl-CoA dehydrogenase, acyl-CoA oxidase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase) dose-dependently. In general, significant increases in the activities of carnitine palmitoyltransferases and enzymes involved in the fatty acid  $\beta$ -oxidation cycle were observed when triacylglycerol was replaced by diacylglycerol to supply more than 65.7 g fatty acids/kg of the diet.

Table 4. Rate of oxidation of palmitoyl-CoA substrate (nmol/min per mg protein) in homogenates of the livers of rats fed on diets containing different proportions of diacylglycerol and triacylglycerol\*

(Mean values and standard deviations)

Dietary fatty acids as diacylglycerol/ triacylglycerol (g/kg diet)	n	Rate of oxidation of palmitoyl-CoA			
		Mitochondrial		Peroxisomal	
		Mean	SD	Mean	SD
<b>Expt 1</b>					
0/93.9	8	1.63 <sup>a</sup>	0.16	3.51 <sup>a</sup>	0.23
93.9/0	8	2.07 <sup>b</sup>	0.20	5.13 <sup>b</sup>	0.45
Pooled SD			0.18		0.36
df			14		14
<b>Expt 2</b>					
0/93.9	7	2.07 <sup>a</sup>	0.13	3.33 <sup>a</sup>	0.28
28.2/65.7	7	2.12 <sup>a</sup>	0.32	3.63 <sup>a</sup>	0.64
65.7/28.2	7	2.49 <sup>b</sup>	0.37	4.35 <sup>b</sup>	0.51
93.9/0	7	2.66 <sup>b</sup>	0.17	4.98 <sup>b</sup>	0.61
Pooled SD			0.27		0.53
df			24		24

<sup>a,b</sup>Mean values within a column within each experiment with unlike superscript letters were significantly different,  $P < 0.05$ .

\*For details of diets and procedures, see pp. 108–111

We also measured the activities of auxiliary enzymes (2,4-dienoyl-CoA reductase and  $\Delta^3, \Delta^2$ -enoyl-CoA isomerase) involved in the pathway to degrade unsaturated fatty acids having *cis* double bond(s) via the  $\beta$ -oxidation pathway to yield acetyl-CoA. Dietary diacylglycerol also increased the activities of these auxiliary enzymes. The extent of the increase was more prominent in reductase than in isomerase.

The activities of marker enzymes for cell organelles (succinate dehydrogenase, catalase, NADPH-cytochrome C reductase and lactate dehydrogenase as markers for mitochondria, peroxisomes, microsomes and cytosols respectively) were measured in Expt 1 but no significant differences between rats fed on triacylglycerol and diacylglycerol were found in the activities of these enzymes (results not shown). In Expt 2 we measured the activity of a marker enzyme for mitochondria (succinate dehydrogenase) using both the 500 g supernatant fraction and isolated mitochondria as the enzyme sources. The activity of the enzyme measured in the 500 g supernatant fraction was comparable among groups (51.6–57.5 nmol/min per mg protein). Values obtained in isolated mitochondria (269–293 nmol/min per mg protein) were approximately 5–6 times higher than those measured in the 500 g supernatant fractions. The activity of succinate dehydrogenase recovered in isolated mitochondrial fraction accounted for 77–81 % of that found in the 500 g supernatant fraction among groups (differences were not statistically significant).

The replacement of dietary triacylglycerol with diacylglycerol increased the proportion of linoleic acid accompanying the decrease in the proportion of oleic acid in liver triacylglycerol in both Expts 1 and 2 (Table 6). Dietary diacylglycerol did not alter the proportion of linoleic acid but increased that of a derivative (arachidonic acid) in liver phospholipid in both Expts 1 and 2. Also, proportions of docosapentaenoic and docosahexaenoic acids in liver phospholipid were slightly but significantly higher in rats fed on the diet containing acylglycerol exclusively as diacylglycerol than in those fed on



Table 5. Expt 2. Activities of enzymes of fatty acid oxidation (nmol/min per mg protein) in the livers of rats fed on diets containing different proportions of diacylglycerol and triacylglycerol\*  
(Mean values and standard deviations for seven observations)

Enzyme activities	Dietary fatty acids as diacylglycerol/triacylglycerol (g/kg diet)											
	0/93.9			28.2/65.7			65.7/28.2			93.9/0		
	Mean	SD	df	Mean	SD	df	Mean	SD	df	Mean	SD	df
Carnitine palmitoyltransferase †† (EC 2.3.1.21)	5.33 <sup>a</sup>	0.40	24	5.48 <sup>a</sup>	0.53	24	6.36 <sup>b</sup>	0.51	24	6.60 <sup>b</sup>	0.17	24
Carnitine palmitoyltransferase †	17.0 <sup>ab</sup>	1.8	24	16.8 <sup>a</sup>	2.0	24	20.3 <sup>bc</sup>	4.0	24	22.6 <sup>c</sup>	3.5	24
Acyl-CoA dehydrogenase (EC 1.3.99.3)	46.8 <sup>a</sup>	3.3	24	48.7 <sup>ab</sup>	7.2	24	53.5 <sup>b</sup>	6.6	24	56.3 <sup>c</sup>	2.8	24
Acyl-CoA oxidase (EC 1.3.3.6)	4.49 <sup>a</sup>	0.57	24	5.10 <sup>a</sup>	0.32	24	6.36 <sup>b</sup>	0.96	24	6.63 <sup>b</sup>	0.86	24
Enoyl-CoA hydratase (EC 4.2.1.17)	2763 <sup>a</sup>	63	24	2860 <sup>ab</sup>	441	24	3239 <sup>bc</sup>	495	24	3351 <sup>c</sup>	186	24
3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)	277 <sup>a</sup>	41	24	291 <sup>a</sup>	66	24	356 <sup>b</sup>	68	24	368 <sup>b</sup>	33	24
2,4-Dienoyl-CoA reductase (EC 1.3.1.34)	8.56 <sup>a</sup>	0.84	24	10.4 <sup>a</sup>	4.5	24	14.7 <sup>b</sup>	3.2	24	17.8 <sup>b</sup>	3.5	24
$\Delta^3, \Delta^2$ -Enoyl-CoA isomerase (EC 5.3.3.8)	47.3 <sup>a</sup>	5.3	24	48.8 <sup>a</sup>	7.3	24	59.5 <sup>ab</sup>	14.7	24	71.1 <sup>b</sup>	11.8	24

<sup>a,b,c</sup>Mean values within a row not sharing a common superscript letter were significantly different,  $P < 0.05$ .

\*For details of diets and procedures, see pp. 108–111.

†Carnitine palmitoyltransferase activity measured radiochemically using freshly isolated mitochondria.

‡Carnitine palmitoyltransferase activity measured spectrophotometrically using a Triton-solubilized preparation of isolated mitochondria.

Table 6. Fatty acid compositions (g/100 g total fatty acids) of liver triacylglycerol and phospholipid in rats fed on diets containing different proportions of diacylglycerol and triacylglycerol

		(Mean values and standard deviations)																				
		Fatty acids																				
		16:0		16:1		18:0		18:1		18:2		18:3		20:4		20:5		22:5		22:6		
Dietary fatty acids diacylglycerol/ triacylglycerol (g/kg diet)	n	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
<b>Expt 1</b>																						
Triacylglycerol																						
0/93.9	7	40.7 <sup>b</sup>	4.0	2.1 <sup>a</sup>	0.4	2.3 <sup>a</sup>	0.3	49.4 <sup>b</sup>	3.3	4.7 <sup>a</sup>	1.5	0.6 <sup>a</sup>	0.3	0.1 <sup>a</sup>	0.1	0.1 <sup>a</sup>	0.0	0.1 <sup>a</sup>	0.0	0.1 <sup>a</sup>	0.0	tr
93.9/0	7	37.2 <sup>a</sup>	2.2	2.1 <sup>a</sup>	0.3	2.2 <sup>a</sup>	0.3	44.2 <sup>a</sup>	2.8	13.1 <sup>b</sup>	3.1	0.6 <sup>b</sup>	0.2	0.4 <sup>a</sup>	0.3	0.2 <sup>a</sup>	0.1	0.2 <sup>a</sup>	0.1	0.2 <sup>a</sup>	0.2	tr
Pooled SD		3.2		0.3		0.3		3.1		2.4		0.3		0.3		0.1		0.1		0.2		
df		12		12		12		12		12		12		12		12		12		12		12
Phospholipid																						
0/93.9	8	20.4 <sup>a</sup>	1.3	0.5 <sup>a</sup>	0.2	24.2 <sup>b</sup>	1.1	9.0 <sup>b</sup>	0.6	11.2 <sup>a</sup>	0.8	0.2 <sup>a</sup>	0.1	22.4 <sup>a</sup>	1.7	0.9 <sup>a</sup>	0.3	1.3 <sup>a</sup>	0.2	9.9 <sup>a</sup>	0.2	0.9
93.9/0	6	19.3 <sup>a</sup>	2.6	0.6 <sup>a</sup>	0.2	21.4 <sup>a</sup>	1.2	7.6 <sup>a</sup>	0.7	12.0 <sup>a</sup>	1.4	0.2 <sup>a</sup>	0.1	26.1 <sup>b</sup>	2.5	0.9 <sup>a</sup>	0.6	1.2 <sup>a</sup>	0.2	10.9 <sup>a</sup>	0.2	1.6
Pooled SD		2.0		0.2		1.1		0.6		1.1		0.1		2.1		0.5		0.2		1.3		1.3
df		12		12		12		12		12		12		12		12		12		12		12
<b>Expt 2</b>																						
Triacylglycerol																						
0/93.9	6	45.5 <sup>a</sup>	1.2	2.0 <sup>a</sup>	0.3	3.0 <sup>a</sup>	0.3	42.0 <sup>b</sup>	1.6	6.3 <sup>a</sup>	1.9	0.7 <sup>a</sup>	0.1	0.2 <sup>a</sup>	0.1	0.1 <sup>a</sup>	0.0	0.1 <sup>a</sup>	0.0	0.1 <sup>a</sup>	0.0	tr
28.2/65.7	5	45.8 <sup>a</sup>	2.3	2.0 <sup>a</sup>	0.3	3.7 <sup>ab</sup>	0.7	39.4 <sup>ab</sup>	3.8	8.1 <sup>a</sup>	1.2	0.6 <sup>a</sup>	0.1	0.2 <sup>a</sup>	0.1	0.1 <sup>a</sup>	0.1	0.1 <sup>a</sup>	0.1	0.1 <sup>a</sup>	0.0	tr
65.7/28.2	7	43.0 <sup>a</sup>	4.9	2.1 <sup>a</sup>	0.3	4.3 <sup>b</sup>	1.0	39.2 <sup>ab</sup>	3.2	10.3 <sup>b</sup>	1.6	0.6 <sup>b</sup>	0.1	0.2 <sup>a</sup>	0.1	0.1 <sup>a</sup>	0.0	0.1 <sup>a</sup>	0.0	0.1 <sup>a</sup>	0.0	tr
93.9/0	7	43.3 <sup>a</sup>	3.7	2.1 <sup>a</sup>	0.1	4.3 <sup>b</sup>	1.4	37.0 <sup>a</sup>	2.7	12.1 <sup>b</sup>	2.2	0.7 <sup>a</sup>	0.1	0.3 <sup>a</sup>	0.0	0.1 <sup>a</sup>	0.1	0.2 <sup>a</sup>	0.1	0.2 <sup>a</sup>	0.1	tr
Pooled SD		3.5		0.2		1.0		2.9		1.8		0.1		0.1		0.1		0.0		0.0		
df		21		21		21		21		21		21		21		21		21		21		21
Phospholipid																						
0/93.9	5	22.8 <sup>b</sup>	0.9	0.6 <sup>a</sup>	0.2	23.6 <sup>b</sup>	1.8	11.0 <sup>a</sup>	3.2	10.3 <sup>a</sup>	1.6	0.4 <sup>ab</sup>	0.1	21.0 <sup>a</sup>	1.8	0.3 <sup>a</sup>	0.1	0.9 <sup>a</sup>	0.0	9.1 <sup>a</sup>	0.0	0.5
28.2/65.7	5	22.8 <sup>b</sup>	2.4	0.5 <sup>a</sup>	0.1	24.5 <sup>b</sup>	2.7	9.1 <sup>a</sup>	0.5	10.7 <sup>a</sup>	0.8	0.3 <sup>a</sup>	0.1	20.7 <sup>a</sup>	1.9	0.4 <sup>a</sup>	0.2	0.9 <sup>a</sup>	0.1	10.0 <sup>a</sup>	0.1	0.8
65.7/28.2	5	22.0 <sup>b</sup>	1.4	0.6 <sup>a</sup>	0.2	20.2 <sup>a</sup>	1.3	8.7 <sup>a</sup>	0.7	11.2 <sup>a</sup>	0.8	0.6 <sup>c</sup>	0.0	24.4 <sup>b</sup>	1.1	0.5 <sup>a</sup>	0.3	1.1 <sup>b</sup>	0.1	10.9 <sup>b</sup>	0.1	0.7
93.9/0	5	19.8 <sup>a</sup>	0.7	0.5 <sup>a</sup>	0.3	19.8 <sup>a</sup>	1.2	9.1 <sup>a</sup>	0.7	11.4 <sup>a</sup>	0.5	0.5 <sup>bc</sup>	0.0	26.4 <sup>c</sup>	1.1	0.4 <sup>a</sup>	0.2	1.2 <sup>b</sup>	0.1	11.0 <sup>b</sup>	0.1	0.4
Pooled SD		1.5		0.2		1.8		1.7		1.0		0.1		1.5		0.2		0.1		0.6		0.6
df		16		16		16		16		16		16		16		16		16		16		16

tr, trace.  
<sup>a,b,c</sup>Mean values within each experiment not sharing a common superscript letter were significantly different,  $P < 0.05$ .  
 \*For details of diets and procedures, see pp. 108–111.

the diet without diacylglycerol or the diet containing 23.2 g fatty acids as diacylglycerol in Expt 2. No such differences were, however, detected in Expt 1. On the contrary, dietary diacylglycerol decreased the proportions of saturated fatty acids (palmitic and stearic acids) and oleic acid in the phospholipid fraction both in Expts 1 and 2.

#### DISCUSSION

The effect of dietary diacylglycerol in lowering serum triacylglycerol (Hara *et al.* 1993) was confirmed in the present study regardless of the feeding period (2 and 3 weeks for Expts 1 and 2 respectively). Moreover, the present study provided evidence that the alterations in the rates of fatty acid synthesis and oxidation in the liver, in addition to the modification in chylomicron assembly and secretion in the small intestine (Murata *et al.* 1994) are the factors by which dietary diacylglycerol reduces concentrations of triacylglycerol in serum. Dietary diacylglycerol also decreased the concentration of triacylglycerol in the liver, but increased that of cholesterol in this tissue in the present study. Thus, there is the possibility that dietary diacylglycerol not only modifies the rates of synthesis and degradation of fatty acid but also those of cholesterol in the liver.

Evidence supports the idea that the rates of fatty acid synthesis and oxidation in the liver are inversely related to each other under various nutritional and pathological conditions. Fasting (Ide *et al.* 1980) and experimental diabetes (Nepokroeff *et al.* 1974) reduce the rate of fatty acid synthesis but increase the rate of fatty acid oxidation in the liver (Mayes & Felts, 1967; Van Harken *et al.* 1969). Dietary fish oil decreases the rate of  $^3\text{H}_2\text{O}$  incorporation into fatty acids but increases ketone body production in the perfused rat liver (Wong *et al.* 1984). A wealth of studies also indicate that fish oil increases the rate of fatty acid oxidation in rat liver peroxisomes (Halminski *et al.* 1991; Rustan *et al.* 1992). These nutritional and pathological conditions all accompany the reduction in the rate of VLDL production in the liver (Mayes & Felts, 1967; Van Harken *et al.* 1969; Wong *et al.* 1984). Conversely, feeding (Ide *et al.* 1980) and treatment of diabetes by insulin (Nepokroeff *et al.* 1974) enhance the rate of fatty acid synthesis but decrease the rate of fatty acid oxidation accompanying the increase in the rate of VLDL production in the liver (Mayes & Felts, 1967; Van Harken *et al.* 1969). In the present study, reciprocal responses in enzymes of fatty acid synthesis and oxidation in the liver were observed between rats fed on diacylglycerol and triacylglycerol. It is therefore probable that dietary diacylglycerol compared with triacylglycerol modifies rates of fatty acid synthesis and oxidation *in vivo* in the liver and these changes contribute to the serum triacylglycerol-lowering effect of this dietary lipid through the modification of the rate of lipoprotein production by the liver.

We measured the rates of mitochondrial and peroxisomal  $\beta$ -oxidation of palmitoyl-CoA using the 500 g supernatant fraction of liver homogenates under the respective conditions for these pathways established by Mannaerts *et al.* (1979) in the present study. They found that mitochondrial activity was stimulated by albumin, but that it severely inhibited peroxisomal activity. We therefore did not include albumin in the measurement of peroxisomal activity but it was added to the assay medium (7.2 mg/ml) to measure mitochondrial activity. Under these conditions, Mannaerts *et al.* (1979) reported that peroxisomal activity was three times higher than mitochondrial activity in rats fed on a laboratory chow. Clofibrate feeding progressively increased peroxisomal activity so that it became ten times higher than the mitochondrial activity in rats fed on this drug (Mannaerts *et al.* 1979). The result of their experiment using isolated hepatocytes, however, indicated that the contribution of peroxisomes to fatty acid oxidation was less than 10 % in both cells from control and clofibrate-fed rats. Although peroxisomal activity measured in the

absence of albumin was also considerably higher than mitochondrial activity measured in the presence of albumin (7.2 mg/ml) in various groups of rats in the present study, it should be stated that the rate of peroxisomal and mitochondrial oxidation of fatty acid in the liver homogenates under the conditions employed in the present study does not necessarily represent the actual *in vivo* rate in these cell organelles. Dietary diacylglycerol compared with triacylglycerol not only increased the rate of oxidation of palmitoyl-CoA substrate in liver homogenates but also increased the activities of various enzymes involved in the  $\beta$ -oxidation of fatty acid. Dietary diacylglycerol did not increase the activities of marker enzymes for mitochondria (succinate dehydrogenase) and peroxisomes (catalase), so this dietary lipid may specifically induce enzymes in the  $\beta$ -oxidation pathway without accompanying the proliferation of these cell organelles.

The degradation of unsaturated fatty acids *via* the  $\beta$ -oxidation pathway to yield acetyl-CoA requires the involvement of several auxiliary enzymes in addition to those required for the  $\beta$ -oxidation of saturated fatty acids (Osmundsen & Hovik, 1988; Schulz, 1991, 1994). 2,4-Dienoyl-CoA reductase and  $\Delta^3, \Delta^2$ -enoyl-CoA isomerase are enzymes located in both mitochondria and peroxisomes (Dommes *et al.* 1981; Käki *et al.* 1987; Palosaari *et al.* 1990; Hakkola *et al.* 1994) and are required for the degradation by the  $\beta$ -oxidation pathway of unsaturated fatty acids having double bonds at even-numbered and odd-numbered positions respectively (Dommes *et al.* 1981; Käki *et al.* 1987; Osmundsen & Hovik, 1988; Schulz, 1991, Hakkola *et al.* 1994). It has been reported that experimental conditions which induce the  $\beta$ -oxidation pathway, such as diabetes (Osmundsen & Björnstad, 1985) and the administration of clofibrate (Dommes *et al.* 1981; Hakkola *et al.* 1994), and partially hydrogenated marine oil (Borrebaek *et al.* 1980) increased the activity of 2,4-dienoyl-CoA reductase in the rat liver. Also, there is evidence to indicate that clofibrate feeding increases  $\Delta^3, \Delta^2$ -enoyl-CoA isomerase activity in both mitochondria and peroxisomes (Palosaari *et al.* 1990; Hakkola *et al.* 1994). In the present study, dietary diacylglycerol compared with triacylglycerol not only increased the activities of carnitine palmitoyltransferase and various enzymes involved in the  $\beta$ -oxidation cycle but also those of 2,4-dienoyl-CoA reductase and  $\Delta^3, \Delta^2$ -enoyl-CoA isomerase. These auxiliary enzymes may therefore be controlled in coordination with other  $\beta$ -oxidation enzymes under various nutritional and pathological conditions.

Dietary diacylglycerol compared with triacylglycerol significantly modified the fatty acid compositions of triacylglycerol and phospholipid in the liver in spite of the fact that the fatty acid compositions of these dietary lipids were comparable. The reduction by dietary diacylglycerol in the rate of fatty acid synthesis as expected from reductions in the activities of enzymes in this pathway may increase the relative availability of linoleic acid of dietary origin as the substrate for enzymes involved in glycerolipid biosynthesis and in fatty acid desaturation and elongation. This consequence may increase the proportion of linoleic acid in liver triacylglycerol and that of arachidonic acid in the phospholipid fraction as observed in rats fed on diacylglycerol in the present study. The proportions of derivatives of  $\alpha$ -linolenic acid (docosapentaenoic and docosahexaenoic acids) also slightly but significantly increased in liver phospholipid of rats fed on diets containing 65.7 and 93.8 g fatty acids/kg in the form of diacylglycerol relative to that in the animals fed on the diet devoid of diacylglycerol in Expt 2. Thus, the relative availability of  $\alpha$ -linolenic acid for enzymes in fatty acid desaturation and elongation may also be augmented in the livers of rats fed on diacylglycerol. The increased availability of polyunsaturated fatty acids relative to other fatty acids through the depression in the rate of fatty acid synthesis may also account for the observed increases in the activities of 2,4-dienoyl-CoA reductase and  $\Delta^3, \Delta^2$ -enoyl-CoA isomerase in the liver of rats fed on diacylglycerol.

Malonyl-CoA mediated inhibition of carnitine palmitoyltransferase represents a mechanism by which mitochondrial oxidation of long-chain fatty acids is regulated (Schulz, 1991). As malonyl-CoA is the enzyme product of acetyl-CoA carboxylase, its concentration in the liver decreases proportionally as the rate of fatty acid synthesis decreases (Boyd *et al.* 1981; Malewiak *et al.* 1985; Schulz, 1991). In the present study, dietary diacylglycerol compared with triacylglycerol at least decreased the activities of some lipogenic enzymes, indicating that this dietary lipid decreased fatty acid synthesis in the liver. Although we did not measure the activity of acetyl-CoA carboxylase in the present study, it is probable that this dietary lipid also decreases the activity of this enzyme in the liver. Thus, it is probable that the dietary diacylglycerol mediated inhibition of fatty acid synthesis accompanies the reduction in malonyl-CoA concentrations in the liver and thus augments fatty acid oxidation more than that expected from the increases in the activities of various enzymes in fatty acid oxidation. Measurements of the activity of acetyl-CoA carboxylase and hepatic concentration of malonyl-CoA in rats fed on diacylglycerol is required to clarify this point.

In conclusion, dietary diacylglycerol compared with triacylglycerol decreased the activities of enzymes of fatty acid synthesis but increased those of enzymes involved in the fatty acid oxidation pathway in rat liver. Thus, alteration in fatty acid metabolism in the liver may be a crucial factor responsible for the serum triacylglycerol-lowering effect of diacylglycerol in the rat.

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