Biochem. J. (1988) 255, 929–935 (Printed in Great Britain)

Secretion and storage of newly synthesized hepatic triacylglycerol fatty acids *in vivo* in different nutritional states and in diabetes

Julia M. DUERDEN and Geoffrey F. GIBBONS

Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, U.K.

Hepatic lipid synthesis was measured in rats *in vivo* with ^3H_2O, and the appearance of label in triacylglycerol and its constituent fatty acid and glycerol moieties was determined. In rats treated with Triton WR1339, the amount of newly synthesized fatty acid secreted as very-low-density lipoprotein (VLDL) triacylglycerol was greater during the dark phase of the diurnal cycle than during the light phase (11.3 versus 4.8 μmol of ^3H_2O/3 h per g of liver respectively). However, the total mass of VLDL triacylglycerol secreted remained constant, as did the amount of label in the secreted triacylglycerol glycerol. Newly synthesized fatty acids comprised only a small proportion of the total VLDL triacylglycerol fatty acids (TGFA) at both times (dark phase, 7.7%; light phase, 2.4%). Starvation for 24 h resulted in a small increase in the secretion of VLDL triacylglycerol. However, the contribution from newly synthesized fatty acids was decreased. Similar effects were observed in streptozotocin-diabetic animals. During the light and dark phases of the cycle, similar quantities of newly synthesized TGFA entered the hepatic cytosol, and these amounts were much smaller than those secreted as VLDL triacylglycerol. The mass of cytosolic triacylglycerol showed a diurnal variation, with a greater concentration during the light phase than in the dark. In diabetes, the mass of triacylglycerol was increased in the cytosol, as was the incorporation of labelled acylglycerol glycerol. Diabetes also abolished the diurnal variation in the quantity of cytosolic triacylglycerol. In each group of animals the specific radioactivity of the microsomal triacylglycerol was similar to that of the respective newly secreted plasma VLDL. The specific radioactivity of the cytosolic triacylglycerol was only 15.8% (dark phase) or 16.8% (light phase) that of the microsomal triacylglycerol. This increased to 35.5% in the starved animals and 40.2% in the diabetic animals.

INTRODUCTION

Hepatic triacylglycerol is synthesized on the cytosolic surface of the endoplasmic reticulum (Bell *et al.*, 1981). This process utilizes either fatty acids synthesized *de novo* within the liver (Windmueller & Spaeth, 1967; Boogaerts *et al.*, 1984) or exogenous preformed fatty acids derived from the blood plasma (Heimberg *et al.*, 1978; Fukuda *et al.*, 1982; Topping & Mayes, 1982) or from incoming lipoproteins (Van Zuiden *et al.*, 1983). Newly synthesized triacylglycerol may be either channelled into the liver cytosol as lipid droplets or packaged into nascent very-low-density lipoprotein (VLDL) for secretion into the plasma (Chao *et al.*, 1986). Little is known of the factors responsible for allocating triacylglycerol into each of these pathways. This information may be important in understanding the causes of the increased hepatic VLDL secretion which contributes to hypertriglyceridaemia in animal models (Schonfeld & Pfleger, 1971; Azain *et al.*, 1985) and in man (Reaven & Greenfield, 1981).

Previous studies using the isolated perfused liver model have investigated the source of the fatty acid utilized for VLDL triacylglycerol synthesis (Fukuda *et al.*, 1982). However, to our knowledge, no similar studies have been carried out on animals in different nutritional states *in vivo*. Neither is there any information concerning the fate of triacylglycerol fatty acid (TGFA) synthesized *de novo* as regards the question of whether nutritional manipulations and diabetes affect the proportion entering the cytosolic or secretory (VLDL) pathways. The major objective of the present work was to establish a model *in vivo* for studying these questions by using ^3H_2O as the fatty acid precursor. This technique has been used by several workers to measure rates of hepatic fatty acid synthesis *in vivo* (Hems *et al.*, 1975; Stansbie *et al.*, 1976; Munday & Williamson, 1983; Schofield *et al.*, 1987), but in most cases triacylglycerol synthesis, specifically, was not investigated. Meaningful interpretation of such data obviously requires knowledge of the relative labelling of the fatty acid and glycerol moieties. These relative amounts of labelled glycerol may also provide useful information about the utilization of unlabelled (i.e. not synthesized *de novo*) fatty acids for hepatic triacylglycerol synthesis, as shown previously for adipose tissue (Brooks *et al.*, 1982, 1983). We have applied the ^3H_2O-incorporation technique to investigate the above parameters of hepatic triacylglycerol metabolism mainly during the pronounced nutritional variation which occurs over the diurnal cycle in rats (Scott & Potter, 1970; Munday & Williamson, 1983; Gibbons *et al.*, 1984; Fukuda *et al.*, 1985), during starvation and after induction of streptozotocin-diabetes.

MATERIALS AND METHODS

Maintenance of animals

Male Wistar rats were housed individually in plastic wire-bottomed cages at an ambient temperature of 22±2 °C in a windowless room which was lit artificially

Abbreviations used: VLDL, very-low-density lipoprotein; TGFA, triacylglycerol fatty acids.

between 08:00 and 20:00 h (schedule A) or between 16:00 and 04:00 h (schedule B). The animals were fed on a commercially available pelleted diet (Diet 41B; Dixon and Sons, Ware, Herts., U.K.), with the following composition (by wt.): 46.7% carbohydrate (mainly starch), 15.4% protein, 3.1% fat and 4.4% crude fibre. Food and water were available *ad libitum* to all groups except the starved animals, which were taken from lighting schedule B and denied food for 24 h before experimentation. In some experiments animals from both lighting schedules were made diabetic by injecting streptozotocin (75 mg/kg body wt.) into the tail vein under light diethyl ether anaesthesia. Streptozotocin was dissolved in 0.01 M-citrate buffer, pH 4.5, on the day of use. Hypoglycaemia occurs 7 h after injection of the drug (Schein *et al.*, 1971). To counteract this effect, rats were given glucose via their drinking water between 7 and 20 h after streptozotocin treatment. To assess diabetic state, a urine sample was checked for glucose concentration with Diabur-Test strips (Boehringer Mannheim). In all animals used, blood glucose concentrations were greater than 18 mM. Animals were used 2–3 days after streptozotocin treatment. Groups of rats were taken for experiments at the mid-point of the light phase (schedule A) and at the mid-point of the dark phase (schedule B) on the same day. These groups were denoted L_6 and D_6 respectively. All animals were maintained on these lighting schedules for at least 2 weeks before the experiment started. At these time points in the diurnal cycle, the different groups of animals weighing 240–270 g were injected with 5 mCi of ^3H_2O intraperitoneally in a total volume of 0.2 ml. In some cases animals received 1 ml of 10% (w/v) Triton WR1339 (polymeric *p*-isooctylpolyoxyethylenephenol) in 0.9% NaCl via the tail vein under light anaesthesia and were simultaneously injected intraperitoneally with 3 mCi of ^3H_2O. At 1 h after injection of ^3H_2O alone or 3 h after injection of Triton WR1339, together with ^3H_2O, animals were anaesthetized with sodium pentobarbital. Blood was collected from the descending vena cava, and the liver was excised. One part was placed immediately on ice and another part was frozen in liquid N_2. The ice-chilled livers were homogenized in 10 vol. of 0.25 M-sucrose with a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 3000 rev./min (800 *g*) for 15 min to sediment the nuclei and cell debris. The resulting supernatant was centrifuged at 14000 rev./min (16000 *g*) for 20 min to sediment the mitochondria; both spins were carried out in a Hi-Spin 21 MSE 8 × 50 rotor. The supernatant from the latter spin was centrifuged at 40000 rev./min (106000 *g*) for 1 h in a fixed-angle rotor in a Beckman ultracentrifuge to sediment the microsomal fraction. The microsomal pellet was washed by resuspension in 0.25 M-sucrose and re-centrifugation, thus decreasing contamination of the microsomal fraction with cytosolic triacylglycerol to <0.2%. This was assessed in a separate experiment by measuring the recovery of a [^{14}C]triacylglycerol internal standard (added to the original homogenate) from the microsomal pellet.

Washed microsomal pellets were sonicated in 1 ml of distilled water at 4 °C for 3 min.

Isolation of plasma VLDL

Venous blood was centrifuged to obtain the plasma. Plasma (1.0 ml) was layered under a solution of density 1.006 g/ml (containing 11.4 g of NaCl, 0.1 g of EDTA and 0.1 g of NaN_3 per litre) and centrifuged at 20000 rev./min in a Beckman ultracentrifuge SW-50.1 rotor for 30 min. The floating chylomicrons were removed. The samples after chylomicron removal were further centrifuged at 40000 rev./min in the same rotor for 18 h to isolate the floating VLDL, which was obtained by tube slicing.

Lipid analysis

Total lipid was extracted from the plasma VLDL, whole liver, cytosolic and microsomal fractions by the method of Folch *et al.* (1957). To adjust for losses of triacylglycerol during the extraction procedure, an internal standard of [^{14}C]triacylglycerol was added. Triacylglycerol mass was measured by the glycerol-phosphate oxidase method (Trinder, 1969) with a kit from Boehringer Mannheim. Incorporation of ^3H_2O into the triacylglycerol was measured by t.l.c. of the lipid extract on silica gel G, with hexane/diethyl ether/acetic acid (35:15:1, by vol.) as the developing solvent. Plates were air-dried and sprayed with Rhodamine 6G for identification. The triacylglycerol band was eluted with diethyl ether, and a portion was removed for dual-label scintillation counting (^{14}C and ^{3}H).

Estimation of ^3H_2O incorporation into the fatty acyl and glycerol moieties of plasma VLDL and cytosolic triacylglycerol

The triacylglycerol of the plasma VLDL and cytosol after t.l.c. was eluted from the silica gel with peroxide-free ether. The ether was evaporated with O_2-free N_2, and a sample of the residue was removed for measurement of radioactivity. The remainder of the labelled triacylglycerol eluted from the t.l.c. plate was saponified with 7.5% (w/v) KOH in ethanol (10 ml) at 70 °C for 1 h. To the hydrolysate was added distilled water (10 ml) and 12 M-HCl to give pH 1–2. The fatty acids were extracted into hexane (10 ml). After evaporation of the hexane, the labelled fatty acid fraction was dissolved in ethanol (1 ml) before addition of scintillant. Samples were assayed for ^{3}H and ^{14}C. Subtraction of the ^{3}H content of the fatty acid fraction from that of the total triacylglycerol gave the [^{3}H]glycerol content.

Other analytical procedures

Plasma glucose was measured by the method of Slein (1963). Protein was assayed by the method of Lowry *et al.* (1951). The specific radioactivity of the plasma water was determined by scintillation counting.

Calculation of the mass of fatty acids synthesized

Incorporation of 1 μmol of ^3H_2O into fatty acids is equivalent to the incorporation of 1.74 μg-atoms of carbon from all sources (Jungas, 1968). This relationship was used to calculate the mass of fatty acids synthesized (1 μmol of C_{18} fatty acid = 10.34 μmol of ^3H_2O).

Materials

Triton WR1339 and streptozotocin were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Radiochemicals were obtained from Amersham International, Little Chalfont, Bucks., U.K. The sources of other materials used in this work have been described previously (Gibbons *et al.*, 1986).

Table 1. Plasma VLDL triacylglycerol concentration in rats treated with Triton WR1339

Rats were injected with Triton WR1339 (intravenously) followed by ^3H_2O (intraperitoneally). After 3 h, blood was sampled from the descending vena cava, the plasma VLDL was isolated and its triacylglycerol content determined. The values in the Table represent means ± S.E.M., with the numbers of animals in parentheses. Values marked * and ** are significantly different from those observed in the D_6 fed animals at $P < 0.05$ and $P < 0.01$ respectively.

State of animals	VLDL triacylglycerol (mg/ml of plasma)	VLDL triacylglycerol [mg/g of liver (output)]
Fed (D_6)	4.77 ± 0.36 (5)	4.24 ± 0.28 (5)
Fed (L_6)	6.11 ± 0.89 (5)	5.70 ± 0.84 (5)
Starved (24 h) (D_6)	5.26 ± 0.39 (4)	6.16 ± 0.48 (4)**
Diabetic (D_6)	6.69 ± 0.57 (5)*	6.24 ± 0.53 (5)**

RESULTS

Hepatic VLDL triacylglycerol secretion in rats treated with Triton WR1339

At 3 h after injection of Triton WR1339, the light-phase and dark-phase animals contained 6.11 ± 0.89 and 4.77 ± 0.36 mg of VLDL triacylglycerol/ml of plasma respectively. Assuming a plasma volume of 4.1% of body wt. (Otway & Robinson, 1967) and a measured liver/body-weight ratio of 0.04, this corresponds to an output of 5.70 and 4.24 mg/g of liver in the light-phase and dark-phase animals (67.8 mg and 49.5 mg per whole liver respectively). In starved animals treated with Triton, the output of VLDL triacylglycerol showed a small but significant increase ($P < 0.01$) (Table 1). A similar increase was observed in animals made insulin-deficient 2 days earlier by injection of streptozotocin. In each case, the plasma VLDL triacylglycerol content of the saline-treated control animals was less than 7% of their Triton-treated counterparts.

In the above experiments, animals were injected with ^3H_2O simultaneously with Triton, and the incorporation of ^3H into the glycerol and fatty acid (TGFA) moieties of the secreted VLDL triacylglycerol were determined. These results are given in Table 2, and show that the labelled TGFA content was lower during the light phase than in the dark phase of the diurnal cycle. Starvation for 24 h or induction of diabetes with streptozotocin resulted in a further decrease in the labelled TGFA. Despite these differences, the content of labelled glycerol was similar in all these circumstances.

The possibility that some of the VLDL triacylglycerol may have been associated with particles large enough to float with the chylomicron fraction was investigated by measuring the ^3H content of the fatty acids derived from the chylomicron triacylglycerol. This was barely measurable in the normal and 24 h-starved animals. In the diabetic animals, this amounted to 20% of the label in the corresponding VLDL TGFA.

By using previously established relationships between carbon and ^3H flux into fatty acids (Jungas, 1968; Brunengraber *et al.*, 1972), it is possible to calculate the mass of VLDL triacylglycerol produced exclusively from newly synthesized fatty acids (see the Materials and methods section). These values have been calculated for each of the states studied and compared with the total amounts of VLDL triacylglycerol secreted in each case (Table 2). The contribution of newly synthesized fatty acid decreased in the order: dark-phase animals > light-phase animals > starved ≡ diabetic. However, even during the dark phase, only a minor proportion of secreted triacylglycerol was derived from newly synthesized fatty acids.

Effects of diurnal variations and diabetes on cytosolic triacylglycerol

The mass and ^3H content of the liver cytosolic triacylglycerol was determined 1 h after injection of ^3H_2O into normally fed animals midway through the dark and light phases of the diurnal cycle. Similar measurements were carried out in diabetic animals.

Table 2. Secretion of labelled hepatic VLDL triacylglycerol in rats treated simultaneously with Triton WR1339 and ^3H_2O

Rats were treated as described in the legend to Table 1. Samples of the purified VLDL triacylglycerol were assayed for ^3H content. The remainder of each sample was saponified and the fatty acid fraction was isolated and assayed for ^3H. The [^3H]glycerol content was determined by subtraction of the labelled fatty acid moiety from that present in the triacylglycerol. All these values were adjusted for recovery of the ^{14}C-labelled internal standard. The quantity of newly synthesized fatty acids (in μmol) secreted as VLDL (column 5) was divided by 3 to give the corresponding values for triacylglycerol. These values were then used to calculate the percentages in column 6. Values are means ± S.E.M., with the numbers of animals in parentheses. Values marked * and *** are significantly different from the fed (D_6) values at $P < 0.05$ and $P < 0.001$ respectively. The M_r of triacylglycerol was 900.

State of animals	VLDL triacylglycerol secreted: Total mass (μmol/g of liver)	Fatty acid (μmol of ^3H_2O/g of liver)	Glycerol (μmol of ^3H_2O/g of liver)	Newly synthesized fatty acids secreted (μmol/g of liver)	Triacylglycerol formed from newly synthesized fatty acids (% of total)
Fed (D_6)	4.71 ± 0.31 (5)	11.32 ± 1.62 (4)	10.20 ± 3.19 (4)	1.09 ± 0.16 (4)	7.7
Fed (L_6)	6.33 ± 0.99 (5)	4.79 ± 1.41 (5)*	12.04 ± 2.87 (5)	0.46 ± 0.14 (5)*	2.4
Starved (24 h) (D_6)	6.84 ± 0.43 (4)*	1.30 ± 0.12 (4)***	10.44 ± 1.41 (4)	0.13 ± 0.01 (4)***	0.6
Diabetic (D_6)	6.93 ± 0.59 (5)*	1.29 ± 0.10 (5)***	7.41 ± 0.48 (5)	0.12 ± 0.01 (5)***	0.6

Table 3. Changes in liver cytosolic triacylglycerol resulting from diurnal variation and streptozotocin-diabetes

Normal animals were injected intraperitoneally with ^3H_2O midway through the light and dark phases of the diurnal cycle. Other animals were made diabetic by tail-vein injection of streptozotocin and were treated with ^3H_2O as above after 3 days. At 1 h after injection of ^3H_2O, the animals were anaesthetized with phenobarbital and the livers were removed. The cytosolic triacylglycerol was isolated, and samples were used for determination of mass and radioactivity. Another portion was hydrolysed, the fatty acids were extracted and the ^3H content was determined. Numbers in parentheses represent the numbers of animals in each group. L_6 values marked *, ** and *** are significantly different from the corresponding D_6 values at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively. Values marked † and †† for the diabetic groups are significantly different from the normal group at the corresponding time of day at $P < 0.05$ and $P < 0.01$ respectively.

Time of day and state of animals	Mass of triacylglycerol in the cytosol (mg/g of liver)	Mass of triacylglycerol in the cytosol (μg/mg of cytosolic protein)	Incorporation of ^3H_2O into TGFA (μmol of ^3H_2O/h per g of liver)	Incorporation of ^3H_2O into glycerol (μmol of ^3H_2O/h per g of liver)
D_6 normal	0.87±0.17 (8)	13.06±2.04 (8)	0.37±0.05 (7)	0.48±0.10 (7)
L_6 normal	1.90±0.26 (7)***	30.74±4.84 (7)**	0.28±0.04 (7)	0.57±0.11 (7)
D_6 diabetic	2.37±0.45 (8)††	47.49±7.36 (8)	0.34±0.05 (4)	1.13±0.25 (4)†
L_6 diabetic	2.44±0.26 (8)	44.58±5.23 (8)	0.16±0.02 (4)*	0.78±0.18 (4)

Table 4. Total hepatic TGFA synthesis and proportion entering plasma VLDL and cytosolic triacylglycerol

Animals were injected with ^3H_2O at the mid-points of the dark and light phases of the diurnal cycle; 1 h later the animals were anaesthetized and the livers removed. From a portion of each liver the triacylglycerol was isolated, hydrolysed, and the radioactivity of the fatty acid fraction was determined. Another portion of each liver was used to isolate the cytosolic triacylglycerol. The fatty acid fraction was again obtained and its ^3H content was measured. These measurements were used to calculate the values in columns 2 and 5, and were derived from the experiment described in Table 3. Another group of animals was injected simultaneously with ^3H_2O and Triton WR1339; 3 h later the animals were anaesthetized and samples of blood removed. Labelled fatty acids were isolated from the plasma VLDL triacylglycerol. These values relate to the experiment described in Table 2 and are expressed as secretion per g of liver per h in column 3. The total amounts of fatty acids synthesized per h by the liver were calculated by adding the amounts remaining in the liver (column 2) to those secreted in the VLDL (column 3). These values were then used to calculate the percentage of the total synthesized fatty acids that was secreted as VLDL (column 4) and the percentage that entered the cytosol (column 6). Values marked * and *** are significantly different from the corresponding values at D_6 at $P < 0.05$ and $P < 0.001$ respectively. Abbreviation: n.d., not determined.

State of animals	Total liver TGFA (μmol of ^3H_2O/h per g of liver)	VLDL TGFA secreted (μmol of ^3H_2O/h per g of liver)	VLDL TGFA secreted (% of total synthesized)	Cytosolic TGFA (μmol of ^3H_2O/h per g of liver)	Cytosolic TGFA (% of total synthesized)
Fed (D_6)	2.83±0.75 (8)	3.77±0.54 (4)	57.1	0.37±0.05 (7)	5.6
Fed (L_6)	1.64±0.29 (8)	1.59±0.47 (5)*	49.2	0.28±0.04 (7)	8.7
Starved (D_6)	0.81±0.08 (8)	0.43±0.04 (4)***	34.7	n.d.	n.d.
Diabetic (D_6)	1.68±0.34 (4)	0.43±0.03 (4)***	20.4	0.34±0.05 (4)	16.1

Table 3 shows that, in the normal animals, the concentration of cytosolic triacylglycerol was significantly higher during the light phase than in the dark phase of the cycle. However, there was no significant difference in the amounts of newly synthesized fatty acid incorporated into the cytosolic triacylglycerol at the two times of day. This contrasted with the different amounts of fatty acid synthesized *de novo* that was incorporated into VLDL triacylglycerol at these times. The liver cytosolic triacylglycerol content of the diabetic animals was significantly higher than normal at the mid-point of the dark phase of the cycle, and the diurnal variation was abolished. However, incorporation of ^3H_2O into cytosolic TGFA in the diabetic animals was greater at the mid-point of the dark phase of the cycle than at the mid-point of the light phase. In these animals, the increase in triacylglycerol mass at D_6 was accompanied by an increase in the incorporation of ^3H_2O into triacylglycerol glycerol compared with that observed in normal animals.

Relative amounts of newly synthesized fatty acid entering the cytosolic and VLDL triacylglycerol

A group of animals was injected simultaneously with Triton and ^3H_2O, and the rate of entry of ^3H-labelled TGFA into the plasma VLDL fraction was determined. Another group of animals was injected with ^3H_2O only, and 1 h later the label associated with the total hepatic TGFA was measured. Of this, the quantity of labelled TGFA appearing in the cytosol was also measured. The sum of the labelled VLDL TGFA in the former group and the hepatic TGFA in the latter should reflect the total quantity of TGFA synthesized by the liver in each of the states examined. It is thus possible to calculate the proportion of this total quantity which enters the

Table 5. Specific radioactivities of liver subcellular and VLDL triacylglycerol fractions

The specific radioactivities of the cytosolic and microsomal triacylglycerol from the liver was determined in rats which had been injected with ^3H_2O. The specific radioactivity of the plasma VLDL triacylglycerol was determined in rats which had received Triton WR1339 in addition to ^3H_2O.

State of animals	Sp. radioactivity (nmol of $^3H_2O/\mu g$ of triacylglycerol)		
	VLDL	Cytosol	Microsomal fraction
Fed (D_6)	4.58±0.46 (5)	0.69±0.07 (8)	4.36±0.96 (8)
Fed (L_6)	2.92±0.55 (5)	0.69±0.10 (8)	4.10±0.64 (8)
Starved (D_6)	1.98±0.15 (4)	0.54±0.13 (8)	1.52±0.30 (8)
Diabetic (D_6)	1.30±0.11 (5)	0.84±0.22 (8)	2.09±0.63 (8)

cytosolic triacylglycerol and that which enters the VLDL. These results are shown in Table 4.

During the dark phase of the cycle, more than 50% of the triacylglycerol produced from newly synthesized fatty acids by the liver had entered the plasma VLDL. This proportion decreased somewhat during the light phase of the cycle. In contrast, the proportion of newly synthesized TGFA entering the cytosolic compartment was much lower, irrespective of the time of day at which the measurements were made (Table 4). In the diabetic animals the proportion of newly synthesized TGFA entering the cytosol increased compared with that of the normal rats.

It remained possible that Triton treatment affected the rate of hepatic triacylglycerol synthesis or secretion. However, Otway & Robinson (1967) have previously shown that, in male rats, Triton did not affect the amounts of triacylglycerol remaining in the liver. In the present work, in which rats were injected with ^3H_2O, there was no significant difference in the amounts of labelled hepatic TGFA in the presence or the absence of Triton treatment (results not shown).

Specific radioactivities of triacylglycerol in hepatic subcellular fractions and in plasma VLDL

Since most, if not all, of the labelled triacylglycerol leaving the liver remains in the plasma as VLDL in the presence of Triton, and since this is relatively undiluted with pre-existing unlabelled VLDL, its specific radioactivity should reflect that of the liver triacylglycerol pool from which it was immediately derived. Accordingly, rats were injected with ^3H_2O, and 1 h later the liver was removed. A portion was homogenized and fractionated into the low-speed pellet, mitochondria, microsomal fraction and cytosol. Triacylglycerol was extracted and purified from the last two fractions, and the specific radioactivities are given in Table 5. In another group of rats, the specific radioactivities of the plasma VLDL triacylglycerol were determined after simultaneous injection of Triton and ^3H_2O. In the normal fed animals, at the mid-point of the dark phase of the diurnal cycle, the specific radioactivities of the plasma VLDL and microsomal triacylglycerol were almost identical. During the light phase the former was 71% of the latter. The specific radioactivity of the cytosolic triacylglycerol was relatively low in each case and did not exceed 17% of that of the corresponding microsomal material. In the starved animals, the decrease in the specific radioactivity of the VLDL triacylglycerol was accompanied by a similar decline in that of the microsomal material. In the diabetic animals, the specific radioactivity of the microsomal triacylglycerol was somewhat higher than that of the VLDL. In this case there was a closer similarity between the specific radioactivities of the VLDL and the hepatic cytosolic triacylglycerol.

DISCUSSION

Source of fatty acid for VLDL triacylglycerol synthesis

Pronounced changes in hepatic lipid metabolism result from changes in food intake and in diabetes. One of the most striking of these is the decrease in the rate of fatty acid synthesis *de novo* which occurs during the light phase of the diurnal cycle, when rats consume little food (Scott & Potter, 1970; Gibbons *et al.*, 1984; Fukuda *et al.*, 1985), during longer periods of starvation (Boyd *et al.*, 1981) and in insulin-dependent diabetes (Fain *et al.*, 1965; Amatruda & Chang, 1983). The present work has shown that these decreases were accompanied by corresponding changes in the amounts of newly synthesized fatty acids secreted as VLDL triacylglycerol (Table 2). However, in the normal fed animals, the decrease in newly synthesized fatty acid output during the light phase was not matched by a decline in the total mass of VLDL triacylglycerol secreted (Table 2). Previously, Goh & Heimberg (1979) showed that there was little variation in hepatic VLDL triacylglycerol secretion over the diurnal cycle, and a more recent study (Marrino *et al.*, 1987) failed to show any significant differences in output during the light and dark phases of the cycle. The present work shows small but significant increases in the mass of VLDL triacylglycerol secreted after 24 h starvation and in streptozotocin-diabetes (Table 2). This conclusion was unaffected by the small or negligible amounts of VLDL triacylglycerol which may have been associated with the chylomicron fraction (see the Results section). Otway & Robinson (1967) measured the total amounts of triacylglycerol entering the plasma in the fed and starved states. Their results implied that starvation led to an increased secretion of triacylglycerol from the liver. The increased release of VLDL by the liver during starvation may reflect an increased requirement of extrahepatic tissues for VLDL triacylglycerol in these circumstances. In this respect, it is noteworthy that, during starvation, VLDL triacylglycerol is a major source of energy for muscle tissue (Wolfe & Durkot, 1985). It is possible that the enhanced rate of appearance of VLDL in the plasma in the diabetic animals may have resulted from an increased intestinal contribution (Risser *et al.*, 1978).

The rate of hepatic fatty acid synthesis reaches a peak at the time of maximum food intake over the diurnal cycle (i.e. midway through the dark phase; Fukuda *et al.*, 1985). However, even at this time newly synthesized fatty acids constituted only 7.7% of the total VLDL TGFA secreted *in vivo* (Table 2). A similar proportion (9%) was found by Azain *et al.* (1985) in the isolated perfused liver *in vitro*. In the present work, this proportion declined to 2.4% at L_6 and to 0.6% after starvation or induction of streptozotocin-diabetes (Table 2). It appeared therefore that, even in well-fed animals, VLDL triacylglycerol is

derived mainly from preformed fatty acids either from the plasma or from pre-existing hepatic TGFA (Yamamoto *et al.*, 1987).

Channelling of newly synthesized fatty acid into VLDL and hepatic cytosol

The results of Table 4 show that, when food intake was high (i.e. at D_6), over 50% of the newly synthesized hepatic TGFA was released into the plasma as VLDL. A slightly lower proportion was released when food intake decreased during the diurnal cycle (i.e. at L_6) and after 24 h starvation. This rapid entry of newly synthesized fatty acid into what is considered to be a secretory pool (Chao *et al.*, 1986) is in contrast with the slow rate of appearance of label in the cytosolic TGFA. Triacylglycerol in the cytosol is regarded as a storage pool (Chao *et al.*, 1986; Palmer *et al.*, 1978; Kondrup, 1979), and the present results suggest that triacylglycerol produced from newly synthesized fatty acids are channelled preferentially into VLDL for export rather than into the cytosolic storage pool. This may explain the higher rates of hepatic VLDL secretion when fatty acid synthesis *de novo* increases after feeding sucrose (Boogaerts *et al.*, 1984; Windmueller & Spaeth, 1985; Yamamoto *et al.*, 1987) or in genetic obesity (Schonfeld & Pfleger, 1971; Azain *et al.*, 1985). Furthermore, the absolute quantity of newly synthesized TGFA entering the cytosolic pool was independent of nutritional state, whereas that in the VLDL secretory pool was not (Table 4). The increase in the concentration of cytosolic triacylglycerol in diabetes is also of some interest. Other investigators have observed increased hepatic triacylglycerol concentrations in insulin-deficient diabetes (Woodside & Heimberg, 1976; Murthy & Shipp, 1979).

Subcellular source of VLDL triacylglycerol

In all the states studied, the specific radioactivity of the microsomal triacylglycerol was similar to that of the plasma VLDL triacylglycerol (Table 5). This supports previous suggestions that the endoplasmic-reticulum triacylglycerol is the immediate source of the nascent VLDL secreted into the plasma (Kondrup, 1979; Higgins & Hutson, 1984; Chao *et al.*, 1986).

Significance of [^{3}H]glycerol incorporation into triacylglycerol

Incorporation of ^3H_2O into acylglycerol glycerol in adipose tissue has been used previously as a measure of total fatty acid esterification rate irrespective of their source (Brooks *et al.*, 1982, 1983). In this tissue, the absence of glycerol kinase ensures that changes in the specific radioactivity of glycerol 3-phosphate resulting from possible variations in concentration of unlabelled glycerol do not occur. The presence of this enzyme in the liver, however, makes comparable information more difficult to interpret, owing to possible changes in the specific radioactivity of glycerol 3-phosphate. Nevertheless, the similarity in the rate of incorporation of ^3H_2O into VLDL triacylglycerol glycerol in the D_6, L_6 and 24 h-starved animals (Table 2) is interesting, and this occurred despite large changes in the incorporation of labelled fatty acids. It is possible that these results are fortuitous and arise from changes in the availability of unlabelled glycerol as described above. However, in the starved state, any increase in the amount of hepatic unlabelled glycerol which might be expected to arise from increased lipolysis would tend to decrease the specific radioactivity of glycerol 3-phosphate. Thus, if the absolute rate of fatty acid esterification remained constant, the incorporation of ^3H into acylglycerol glycerol might be expected to decrease. This did not occur (Table 2). It is thus safe to infer that, at L_6 and after starvation, the total quantity of fatty acids esterified in VLDL did not decrease. This was borne out by the measurements of mass output (Table 1). In a similar way, the increased [^{3}H]glycerol content of the cytosolic triacylglycerol in diabetic animals implied a greater rate of esterification of fatty acids from all sources. This was supported by measurement of the mass of cytosolic triacylglycerol. These results suggest that in liver, as well as in adipose tissue, the [^{3}H]glycerol content of newly synthesized triacylglycerol is a fairly accurate approximation of the overall rate of fatty acid esterification.

We thank Dr. D. H. Williamson for many stimulating discussions of this work, and Mrs. M. Barber for typing the manuscript. This work was supported by a grant from the Medical Research Council (M.R.C.) G.F.G. is a member of the External Scientific Staff of the M.R.C.

REFERENCES

Amatruda, J. M. & Chang, C. L. (1983) Metab. Clin. Exp. **32**, 224–229
Azain, M. J., Fukuda, N., Chao, F.-F., Yamamoto, M. & Ontko, J. (1985) J. Biol. Chem. **260**, 174–181
Bell, R. M., Ballas, L. M. & Coleman, R. A. (1981) J. Lipid Res. **22**, 391–403
Boogaerts, J. R., Malone-McNeal, M., Archambault-Schexnayder, J. & Davis, R. A. (1984) Am. J. Physiol. **246**, E77–E83
Boyd, M. E., Albright, E. B., Foster, D. W. & McGarry, J. D. (1981) J. Clin. Invest. **68**, 142–152
Brooks, B. J., Arch, J. R. S. & Newsholme, E. A. (1982) FEBS Lett. **146**, 327–330
Brooks, B. J., Arch, J. R. S. & Newsholme, E. A. (1983) Biosci. Rep. **3**, 263–267
Brunengraber, H., Sabine, J. R., Boutry, M. & Lowenstein, J. M. (1972) Arch. Biochem. Biophys. **150**, 392–396
Chao, F.-F., Stiers, D. L. & Ontko, J. A. (1986) J. Lipid Res. **27**, 1174–1181
Fain, J. N., Scow, R. O. & Vogoiti, E. J. (1965) Endocrinology (Baltimore) **77**, 137–149
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) J. Biol. Chem. **226**, 497–509
Fukuda, N., Azain, M. J. & Ontko, J. A. (1982) J. Biol. Chem. **257**, 14066–14072
Fukuda, H., Katsurada, A. & Iritani, N. (1985) Biochim. Biophys. Acta **835**, 163–168
Gibbons, G. F., Pullinger, C. R. & Björnsson, 'O. G. (1984) J. Lipid Res. **25**, 1358–1367
Gibbons, G. F., Attwell Thomas, C. P. & Pullinger, C. R. (1986) Biochem. J. **235**, 19–24
Goh, E. H. & Heimberg, M. (1979) Biochem. J. **184**, 1–6
Heimberg, M., Goh, E. H., Klausner, H. A., Soler-Argilaga, C., Weinstein, I. & Wilcox, H. G. (1978) Am. Physiol. Soc. Monogr.: Disturbances in Lipid Metabolism, pp. 251–267
Hems, D. A., Rath, E. A. & Verrinder, T. R. (1975) Biochem. J. **150**, 167–173
Higgins, J. A. & Hutson, J. L. (1984) J. Lipid Res. **25**, 1295–1305
Jungas, R. L. (1968) Biochemistry **7**, 3708–3717
Kondrup, J. (1979) Biochem. J. **184**, 63–71

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Marrino, P., Gavish, D., Shafrir, E. & Eisenberg, S. (1987) Biochim. Biophys. Acta **920**, 277–284
Munday, M. R. & Williamson, D. H. (1983) Biochem. J. **214**, 183–187
Murthy, V. K. & Shipp, J. C. (1979) Diabetes **28**, 472–478
Otway, S. & Robinson, D. S. (1967) J. Physiol. (London) **190**, 321–332
Palmer, J. F., Cooper, C. & Shipley, R. A. (1978) Biochem. J. **172**, 219–226
Reaven, G. M. & Greenfield, M. S. (1981) Diabetes **30**, Suppl. **2**, 66–75
Risser, T. R., Reaven, G. M. & Reaven, E. P. (1978) Diabetes **27**, 902–908
Schein, P. S., Alberti, K. G. M. M. & Williamson, D. H. (1971) Endocrinology (Baltimore) **89**, 827–834
Schofield, P. S., Sugden, M. C., Corstorphine, C. G. & Zammit, V. A. (1987) Biochem. J. **241**, 469–474
Schonfeld, G. & Pfleger, B. (1971) Am. J. Physiol. **220**, 1178–1181
Scott, D. F. & Potter, V. R. (1970) Fed. Proc. Fed. Am. Soc. Exp. Biol. **29**, 1553–1559
Slein, M. W. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 117–123, Academic Press, New York and London
Stansbie, D., Brownsey, R. W., Crettaz, M. & Denton, R. M. (1976) Biochem. J. **160**, 413–416
Topping, D. L. & Mayes, P. A. (1982) Biochem. J. **204**, 433–439
Trinder, P. (1969) Ann. Clin. Biochem. **6**, 24–30
Van Zuiden, P. E. A., Erickson, S. K. & Cooper, A. D. (1983) J. Lipid Res. **24**, 418–428
Windmueller, H. G. & Spaeth, A. E. (1967) Arch. Biochem. Biophys. **122**, 362–369
Windmueller, H. G. & Spaeth, A. E. (1985) J. Lipid Res. **26**, 70–81
Wolfe, R. R. & Durkot, M. J. (1985) J. Lipid Res. **26**, 210–217
Woodside, W. F. & Heimberg, M. (1976) J. Biol. Chem. **251**, 13–23
Yamamoto, M., Yamamoto, I., Tanaka, Y. & Ontko, J. A. (1987) J. Lipid Res. **28**, 1156–1165

Received 22 March 1988/14 June 1988; accepted 20 June 1988