Liver Lipid Accumulation and Fatty Acid Flux in Rats Fed a Low Protein Diet Containing Purified Whole Egg Protein[†]

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A time-course experiment related to the incorporation of palmitic acid-1-¹⁴C was performed in growing rats fed a 5 or 20 protein calories percent (PC%) diet containing purified whole egg protein.

No difference was observed with time in the conversion of palmitic acid-1-¹⁴C to respiratory CO₂. Radioactivity present in liver triglycerides (TG) was higher and disappeared more slowly with time in the 5 PC% group than in the 20 PC% group. Conversely, significantly lower radioactivity appeared in serum TG of the 5 PC% group than in that of the 20 PC% group. A marked decrease of serum TG level was observed in the 5 PC% group, which was attributable to a reduced concentration of TG in serum very low density lipoprotein.

From the results obtained, a decreased transport of TG from the liver to blood was considered to be a factor responsible for the liver lipid accumulation in rats fed the low protein diet.

It is well known that lipids accumulate in the liver of animals under various nutritional conditions^{1~5)} or by administration of chemical agents.^{6~8)} Tanaka *et al.*⁹⁾ reported previously that young rats fed a low protein diet containing purified whole egg protein showed specific accumulations of lipids in their livers when the diet contained the protein at 5 PC%. We¹⁰⁾ observed thereafter that the liver TG content for this low protein (5 PC%) diet was twice as much as that for a control (20 PC%) diet, whereas the concentrations of TG and apoprotein in serum VLDL for the low protein diet were significantly lower than those for the control diet when sucrose and 10% soybean oil were used as dietary carbohydrate and fat sources respectively. From these results, an

impaired transport of TG from the liver was suggested to be one of factors responsible for the lipid accumulation. Therefore, we undertook observations on the fatty acid flux *in vivo* through the liver with palmitic acid-1-¹⁴C.

MATERIALS AND METHODS

Experimental animals and diets. Male rats of Wistar strain weighing 40 to 60 g were used. They were housed in individual cages with wire bottoms in a room at a temperature of 22±2°C, a relative humidity of 60±5%, and a 6 a.m. to 6 p.m. light cycle. The rats were fed a commercial stock diet (Type NMF, Oriental Yeast Co., Ltd., Tokyo) until they had reached an average weight of about 75 g. They were divided into two groups of 11 rats each and fed the experimental diets shown in Table I for 23 days. Control rats were fed a 20 PC% diet. For producing rats with fatty livers, a 5 PC% diet was used. Experimental diets and water were given *ad libitum*, but the rats were fasted for about 12 hr before they were sacrificed.

Injection of labeled palmitic acid. Palmitic acid-1-¹⁴C (57.9 mCi/mole, the Radiochemical Centre, Amersham, England) was homogenized in 5% human serum albumin (Fr. V Powder, Fatty Acid Free, Miles Laboratories Inc., Kankakee, USA) in 0.9% NaCl¹¹⁾ and injected into the femoral vein of each rat under light ether anesthesia at a dose of 4.109 μCi/0.4 ml/rat for the 20 PC% group or 2.055 μCi/0.2 ml/rat for the 5 PC% group.

Abbreviations: PC%=protein calories percent, TL=total lipids, TG=triglycerides, PL=phospholipids, FA=fatty acids, FFA=free fatty acids, VLDL=very low density lipoprotein, LDL=low density lipoprotein, LPL=lipoprotein lipase.

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Collection and determination of ^{14}C in the respiratory CO_2 . Immediately after injection, each rat was kept in a glass metabolism apparatus¹²⁾ for 15, 45 and 105 min to determine the radioactivity in the expired CO_2 . Respiratory CO_2 was trapped in a solution of methylcellosolve-monoethanolamine (2:1) mixture for 15, 45 and 105 min. Radioactivity was determined by the method of Jeffay and Alvarez¹³⁾ in an Aloka LSC 651 liquid scintillation spectrophotometer and the correction for quenching was made by the external standard method.

Preparation of serum and tissues. During the next 15 min each rat was taken out of the glass metabolism apparatus, anesthetized by an intraperitoneal injection of nembutal (4 mg/100 g body weight), and had a cannula fixed into the carotid. At the scheduled times, *i.e.*, 30, 60 and 120 min after the injection of palmitic acid-1- ^{14}C , blood was collected from the cannula. An abdominal incision was made and the liver and epididymal adipose tissue were quickly removed, weighed and placed on ice. Serum was separated by centrifugation from the blood samples left to clot at room temperature for 3 hr. Total serum volume was tentatively calculated in accordance with Shiraki's equation.¹⁴⁾

Isolation of serum lipoproteins. Equal amounts of serum from two or three rats of each group were pooled in order to have sufficient materials. Lipoproteins were isolated from each pooled serum by a slight modification of the method described by Lombardi and Ugazio.¹⁵⁾ The saline solutions containing NaCl, NaBr and disodium EDTA were prepared and their density was adjusted by pycnometry to 1.0307, 1.019 and 1.371, respectively. All centrifugations were performed at 16°C in a Hitachi 65P preparative ultracentrifuge using a RP 65 rotor. To each pooled serum (2 to 4 ml) in a polycarbonate (Hitachi 10 PC) tube, saline solution of density 1.0307 (pH 7.4, containing 0.04% disodium EDTA) was added in equal volumes to each pooled serum, and a final volume of 8 ml was attained by adding saline solution of density 1.019 (pH 7.4, containing 0.02% disodium EDTA). The tubes were capped and centrifuged at $105,000 \times g$ for 18 hr. After centrifugation, the floating lipoprotein of $d < 1.019$ (VLDL) that accumulated in the top 1 ml of the tube was removed with the aid of a capillary pipette. To the 7 ml of infranatant solution, 1 ml of saline solution of density 1.371 (pH 7.4, containing 0.02% disodium EDTA) was added. The tubes were capped and centrifuged at $105,000 \times g$ for 24 hr. After centrifugation, the top 2 ml, containing the lipoprotein of $1.019 < d < 1.063$ (LDL), was removed. Both lipoprotein fractions were dialyzed *vs.* 0.9% NaCl solution (pH 7.4, containing 0.02% disodium EDTA) for 24 hr at 4°C.

Lipid analysis and measurement of radioactivity. Serum, serum lipoproteins and tissue TL were ex-

tracted and purified according to the method of Folch *et al.*¹⁶⁾ A pair of epididymal adipose tissues and a representative sample of the liver which was minced and mixed with scissors were homogenized in a mixture of chloroform-methanol (2:1) using a Polytron homogenizer (Kinematica. G.m.b.H., Lucern, Switzerland). The phase containing purified TL was evaporated to dryness. The lipid was redissolved in diethyl ether and suitable portions were applied to thin-layer chromatography on Silica Gel G (Type 60, Merck, Darmstadt, W. Germany) using a solvent system¹⁷⁾ of petroleum ether-diethyl ether-acetic acid (80:20:1). Other suitable portions of the ether extract were transferred into vials as required, and radioactivity present in TL was determined (see below). Compounds were identified with iodine vapor. After the iodine had sublimed, the TG and PL fractions were scraped from the plates and quantitatively transferred into glass test tubes. TG were eluted from the silica gel with diethyl ether, and PL with a solvent mixture of chloroform-methanol-water-formic acid (97:97:4:2) as described by Abramson and Bleicher.¹⁸⁾ The eluates were filtered through Toyo No. 5C paper and portions of the filtrates were transferred to vials. After evaporation of the solvents in a desiccator at reduced pressure, 15 ml of a toluene scintillator¹⁹⁾ was added. Radioactivity was determined as described above. With regard to serum and serum lipoproteins, TG were also determined by the calorimetric method of Van Handel.¹⁹⁾

RESULTS

As shown in Table II, the final body weight of the 5 PC% group was half that of the 20 PC% group. When the liver weight was expressed in values relative to 100 g body weight, the value of the 5 PC% group was slightly, but significantly, higher than that of the 20 PC% group, *i.e.*, the livers of the 5 PC% group were relatively enlarged in comparison with those of the 20 PC% group. In adipose tissue, however, there was no significant difference between relative values for the 20 and 5 PC% groups.

Table III shows the concentrations of TG in whole serum and serum lipoproteins. There was a marked decrease in the level of serum TG in the 5 PC% group which was due to a reduced concentration of this lipid in VLDL.

The respiratory CO_2 was collected for 15, 45 and 105 min after the injection of palmitic acid-1- ^{14}C (Fig. 1). At no time was a significant difference observed in the conversion of

TABLE I. COMPOSITION OF EXPERIMENTAL DIETS

Diet (group)	5 PC% (low protein)	20 PC% (control)
g/100 g of diet		
Sucrose	74.9	59.5
Whole egg protein ^{a)}	5.13	20.5
Soybean oil	10.0	10.0
Cellulose powder ^{b)}	5.00	5.00
Salt mixture ^{c)}	4.00	4.00
Vitamin mixture ^{d)}	0.85	0.85
Choline chloride	0.15	0.15
Metabolizable energy ^{e)} (kcal)	410	410
Protein calories % ^{f)}	5	20

a) Taiyo Foods Co., Ltd., Yokkaichi, Japan.^{b)}

b) Toyo Roshi Co., Ltd., Tokyo, Japan.

c) Tanabe Amino Acid Research Foundation, Osaka, Japan.

d) See. ^{e)} Additionally supplemented with 300 IU retinyl palmitate, 100 IU ergocalciferol and 5 mg *dl*- α -tocopherol per 100 g of diet.

e) Sucrose (g) $\times$ 4 + whole egg protein (g) $\times$ 4 + soybean oil (g) $\times$ 9 (kcal/100 g of diet).

f) Whole egg protein-N $\times$ 6.25 $\times$ 4 $\times$ 100/metabolizable energy.

palmitic acid-1-¹⁴C to expired CO₂.

Figure 2 shows the incorporation of palmitic acid-1-¹⁴C into liver TL, liver and serum PL. Radioactivity present in liver TL was almost the same during the first 60 min but that of the 5 PC% group was significantly higher than that of the 20 PC% group at the 120 min point, which was essentially due to a considerable loss of radioactivity from liver TL of the 20 PC% group but only a small loss from liver TL of the 5 PC% group. No significant difference, however, was observed in the incorporation of palmitic acid-1-¹⁴C into liver PL as time went on. These findings indicate that a small loss of radioactivity from,

FIG. 1. Conversion of Palmitic Acid-1-¹⁴C to Respiratory Carbon Dioxide (CO₂).

Each point represents the mean $\pm$ SEM of three (15 min) or four (45, 105 min) rats.

FIG. 2. Incorporation of Palmitic Acid-1-¹⁴C into Liver Total Lipids (TL), Liver and Serum Phospholipids (PL).

Each point represents the mean $\pm$ SEM of three (30 min) or four (60, 120 min) rats.

a) $P < 0.01$ to 5 PC% group.

TABLE II. DATA ON RATS USED FOR PALMITIC ACID-1-¹⁴C INCORPORATION EXPERIMENT *in vivo*

Each value represents the mean $\pm$ SEM of eleven rats.

Diet (group) PC%	Final body weight g	Body weight gain g/day	Liver weight g/100 g bwt	Epididymal adipose tissue weight g/100 g bwt
20	228.0 $\pm$ 7.5 ^{a)}	6.6 $\pm$ 0.3 ^{a)}	4.24 $\pm$ 0.17 ^{a)}	1.30 $\pm$ 0.09
5	114.6 $\pm$ 5.1	1.6 $\pm$ 0.2	5.55 $\pm$ 0.28	1.17 $\pm$ 0.07

a) $P < 0.005$ to 5 PC% group.

TABLE III. CONCENTRATION OF WHOLE SERUM-
AND SERUM LIPOPROTEIN-TG IN RATS FED
20 OR 5 PC% DIET

Each value represents the mean $\pm$ SEM of eleven (whole serum) or five (VLDL, LDL) samples.

Diet (group) PC%	Triglycerides		
	Whole serum	VLDL mg/100 ml of serum	LDL
20	29.9 $\pm$ 4.1 ^{a)}	26.4 $\pm$ 3.5 ^{a)}	2.3 $\pm$ 0.2
5	10.8 $\pm$ 0.6	7.3 $\pm$ 1.2	2.8 $\pm$ 0.4

^{a)} $P < 0.005$ to 5 PC% group.

or a stagnation of radioactivity in, liver TL of the 5 PC% group is not due to the stagnation of radioactivity in liver PL but to stagnation in other lipid classes of the liver. Radioactivity present in serum PL rose gradually in both groups with time, and during the first 60 min it was somewhat, but not significantly, lower in the 5 PC% group than in the 20 PC% group.

The time curves for radioactivity present in the liver and serum TG, and epididymal adipose tissue TL are shown in Fig. 3. Radioactivity in liver TG was higher at all points, especially significantly higher at the 120 min point, in the 5 PC% group than in the 20 PC% group. As the time proceeded, radioactivity present in liver TG declined in both groups but declined more slowly in the 5 PC% group than in the 20 PC% group. No significant difference was observed in the incorporation of palmitic acid-1-¹⁴C into liver PL (Fig. 2). TG, as well as PL, are the major lipid classes in liver lipids. Therefore, it can be considered that the stagnation of radioactivity in liver TG of the 5 PC% group was reflected in the trend of radioactivity in liver TL of the 5 PC% group. Radioactivity present in serum TG was lower in the 5 PC% group than in the 20 PC% group at all times. Specific radioactivity was also significantly lower during the first 60 min and tended to be lower after 120 min in the 5 PC% group than in the 20 PC% group (Table IV). In adipose tissue TL, in which TG are the major lipid class, no significant difference and no systematic change were observed between the two 20 and 5 PC%

FIG. 3. Incorporation of Palmitic Acid-1-¹⁴C into Liver and Serum Triglycerides (TG), and Epididymal Adipose Tissue Total Lipids (TL).

Each point represents the mean $\pm$ SEM of three (30 min) or four (60, 120 min) rats.

a) $P < 0.01$ to 5 PC% group.

b) $P < 0.005$ to 5 PC% group.

c) $P < 0.1$ to 5 PC% group.

d) $P < 0.02$ to 5 PC% group.

TABLE IV. SPECIFIC RADIOACTIVITY OF SERUM TG
AFTER INJECTION OF PALMITIC ACID-1-¹⁴C INTO
RATS FED 20 OR 5 PC% DIET

Each value represents the mean $\pm$ SEM of three (30 min) or four (60, 120 min) samples.

Diet (group) PC%	Time after injection (min)		
	30	60	120
	dpm/ μ g of TG		
20	44.7 $\pm$ 4.2 ^{a)}	12.0 $\pm$ 1.4 ^{b)}	4.44 $\pm$ 0.43 ^{c)}
5	8.1 $\pm$ 2.9	6.4 $\pm$ 1.6	3.22 $\pm$ 0.39

^{a)} $P < 0.005$ to 5 PC% group.

^{b)} $P < 0.05$ to 5 PC% group.

^{c)} $P < 0.1$ to 5 PC% group.

TABLE V. PERCENTAGE OF RADIOACTIVITY BETWEEN SERUM AND LIVER TG AFTER INJECTION OF PALMITIC ACID-1-¹⁴C INTO RATS FED 20 OR 5 PC% DIET

Each value represents the mean of three (30 min) or four (60, 120 min) samples.

Time after injection min	Diet (group) PC%	Liver TG % of dose	Serum TG	Percentage of serum TG to liver TG %
30	20	7.09	1.541	22.54
	5	9.80	0.125	1.33
60	20	5.93	0.399	10.30
	5	9.06	0.094	1.57
120	20	2.70	0.111	4.41
	5	7.12	0.047	0.69

groups (Fig. 3).

As shown in Table V, the percentage of radioactivity between serum and liver TG was markedly lowered in the 5 PC% group compared with the 20 PC% group at all times. The values for the 5 PC% group were unchanged except for the 120 min value, whereas those of the 20 PC% group dropped rapidly with time.

DISCUSSION

It was found previously¹⁰⁾ that the concentration of FFA in $d > 1.063$ lipoproteins, which contain albumin-FFA complexes, was unchanged between the two 20 and 5 PC% groups. This result rules out the possibility that the accumulation of liver TG in the 5 PC% group is due to increased mobilization of FFA from adipose tissue to the liver.

It appears that the FA oxidation ability is not impaired in the 5 PC% group at the whole animal level (Fig. 1). As pointed out by Yoshida and Harper,¹⁾ the FA oxidation ability in the liver alone cannot be confirmed from the data in experiments on intact animals. In the experiment with the perfused liver, however, no evidence was obtained that the FA oxidation ability is impaired in the 5 PC% group.²⁰⁾ Consequently, it is also unlikely that the accumulation of liver TG in the 5 PC% group results from the impaired FA oxidation ability in the liver.

Decreased transport of TG from the liver to

blood is usually reflected by a lower level of serum TG.^{15, 21)} In the fasting state, the liver is the major source of serum TG.²²⁾ In rats fasted for 12 hr, a marked decrease of serum TG level was observed in the 5 PC% group, which was attributed to a reduced concentration of TG in VLDL (Table III), confirming previous observations.¹⁰⁾ It was also reported previously that the concentration of apoprotein in VLDL was lower in the 5 PC% group than the 20 PC% group.¹⁰⁾ These results, therefore, suggest that the transport of TG from the liver to blood is impaired in the 5 PC% group.

The release of liver TG into the blood has been assessed by a time study of the incorporation of labeled FA into liver and serum (or plasma) TG.^{21, 23)} As shown in Fig. 3, after the injection of palmitic acid-1-¹⁴C, radioactivity present in liver TG was higher and disappeared more slowly with time in the 5 PC% group than in the 20 PC% group. Conversely, lower radioactivity appeared in serum TG of the 5 PC% group than in that of the 20 PC% group. Specific radioactivity of serum TG was also lower at all times in the 5 PC% group than in the 20 PC% group (Table IV). These findings (also see Table V) strongly indicate the impaired transport of liver TG into blood in the 5 PC% group.

In the fasting state,²³⁾ the serum TG level depends mainly on the balance between the release of TG from the liver and the removal of TG-FA in the extrahepatic tissues by the action of LPL. Under these conditions, the activity of LPL in adipose tissue is low and serum TG-FA are directed predominantly towards the muscular tissues and oxidation.²⁴⁾ No difference was observed in the conversion of palmitic acid-1-¹⁴C to respiratory CO₂ between the two 20 and 5 PC% groups at the whole animal level (Fig. 1), and in the perfused liver the ability of this conversion to CO₂ was found not to be impaired in the 5 PC% group compared with the 20 PC% group,²⁰⁾ excluding the possibility that the extrahepatic tissues (mainly muscular tissues) take up and oxidize more serum TG-FA of the 5 PC%

group than of the 20 PC% group. In addition, the incorporation of palmitic acid-1-¹⁴C into adipose tissue TL did not rise significantly in the 5 PC% group (Fig. 3), although the contribution of this tissue towards the removal of TG-FA is minor in the fasting state.²⁴⁾ It appears, therefore, unlikely that the reduction of serum TG level and of incorporation of palmitic acid-1-¹⁴C into serum TG in the 5 PC% group was due to faster utilization of serum TG-FA by the extrahepatic tissues.

Lecithins are the major PL class in the liver,²⁵⁾ as well as in serum.²⁶⁾ In choline deficient rats,^{1,21)} the incorporation of labeled palmitic acid into liver PL was found to be lower than that in control rats but in the present study, no difference was observed between the two 20 and 5 PC% groups, reflecting an adequate supplement of choline in the experimental diets. Measurement of the incorporation of palmitic acid-1-¹⁴C into serum PL provides indirect information on the release of liver TG, since serum PL, as well as TG in the fasting state, are released by the liver. No clear-cut evidence, however, was obtained from the incorporation of palmitic acid-1-¹⁴C into serum PL (Fig. 2) such as that into serum TG (Fig. 3). As described by Lombardi *et al.*,²¹⁾ the major reasons for such a low sensitivity in serum PL are (a) the considerably slower turnover of serum PL, and (b) the fact that among serum lipoproteins, the VLDL fraction has the fastest turnover but the lowest content of PL.

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