Regulation of cholesterol synthesis and storage in fat cells

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Abstract The fat cells of rat epididymal adipose tissue contain an average of 0.5 mg of cholesterol per gram of triglyceride. Of this cholesterol, 90% is nonesterified and 80% is located in the lipid storage compartment. The fat cell cholesterol content correlated positively with cell size. During fasting the free cholesterol of the adipocyte decreased in parallel with triglyceride, whereas the amount of esterified cholesterol did not change. The fat cell cholesterol content is independent of the amount of dietary cholesterol. On in vitro incubation of rat fat cells with radiolabeled acetate, mevalonate, glucose, leucine, or water, labeled cholesterol was synthesized. The rate of cholesterol synthesis increased with fat cell size. Fasting suppressed cholesterol synthesis by 90%, whereas refeeding stimulated the synthesis above values found in normally fed rats. Stimulation of lipolysis with theophylline or with dibutyryl cyclic AMP markedly inhibited cholesterol synthesis in fat cells. Insulin increased the incorporation of glucose and leucine into fat cell cholesterol. The cholesterol synthesis in fat cells was not suppressed by a high cholesterol diet. Addition of very low or low density lipoprotein into the incubation medium suppressed fat cell cholesterol synthesis whereas high density lipoprotein did not. The lipoprotein-free serum stimulated cholesterol synthesis compared with serum-free medium. The rate of cholesterol synthesis in total adipose tissue of rat was estimated to be 4% of that in the liver. It seems unlikely that the increased body cholesterol turnover present in obesity is accounted for by the enhanced cholesterol formation in the enlarged adipose tissue.

Supplementary key words fasting · refeeding · adipose tissue · dietary cholesterol · cholestyramine · obesity · lipoproteins · lipolysis · insulin

Adipose tissue of various mammalian species (1–5), including man (6–10), contains cholesterol that is in dynamic equilibrium with plasma cholesterol (11, 12). Even though the concentration of cholesterol in adipose tissue is quite small (1 mg/g) the storage fat forms one of the largest body cholesterol pools (13), and this tissue may therefore be significant in total body or plasma cholesterol metabolism and turnover. Good evidence for this view is provided by the close correlation that has been demonstrated between adipose tissue mass and total cholesterol turnover in man (14–16).

Little is known about the pathways by which cholesterol enters and leaves the fat cells. Synthesis of cholesterol has been shown to occur at a low rate in adipose tissue of some species (17) but not in others (18). Labeled cholesterol is incorporated in vivo into adipose tissue from injected chylomicrons (19, 20) and lipoproteins (3, 11–13). However, there is no information on the extent to which synthesis, uptake, and storage of cholesterol in fat cells are under metabolic control. Therefore, a systematic series of studies was designed to measure the rate of cholesterol synthesis by rat adipocytes under different experimental conditions.

MATERIALS AND METHODS

Animals

Male rats of the Sprague-Dawley strain, weighing 200–300 g, were used unless otherwise indicated. The animals were housed one per cage in windowless rooms where artificial lights were on from 6 a.m. to 2 p.m. The animals had free access to commercial rat chow (44% carbohydrate, 28% digestible protein, 3.5% fat, vitamins, and salts) and tap water when not given any special diet listed below. The basal chow contained 50 mg of cholesterol and 40 mg of sitosterol per 100 g. To avoid differences due to possible diurnal variations, the rats were killed between 9 and 11 a.m.

Diets and treatments

High sucrose diet. In order to obtain large fat cells for a study of the influence of fat cell size on cholesterol synthesis, rats were given a 15% sucrose solution instead of

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; FFA, free fatty acids; TG, triglyceride; HMG CoA, the coenzyme A ester of β-hydroxy-β-methylglutaric acid; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

drinking water until the age of 23 wk. 1 wk before adipose tissue sampling, water was substituted for the sucrose solution in order to avoid the possible influence of diet on cholesterol synthesis.

High cholesterol diet and cholestyramine. In these experiments, the basal diet was a "fat-free test diet" purchased from Nutritional Biochemicals (Cleveland, Ohio) and supplemented with 10% olive oil. In the high cholesterol diet, cholesterol was dissolved in olive oil and mixed with the solid diet to a final concentration of 5 g/100 g of diet. In the cholestyramine group, 4 g of Cuemid (Merck Sharp & Dohme) was added to 100 g of the basal diet. These diets were fed for 2 wk; during this time the average weight gain was similar (41%) in cholesterol-fed and cholestyramine-fed groups and in controls. Rats fed the high cholesterol diet received 1.2 g of cholesterol per day. The average cholestyramine consumption was 0.89 g/day.

Fasting and refeeding. Rats weighing 204 ± 2 g (mean ± SEM) were used for the study; they were divided at random into three groups. One group received food ad lib., the second group was fasted for 60 hr, and the third group was fasted for 60 hr and subsequently refed for 72 hr. Fasting caused a mean weight reduction of 18%, and during the refeeding period the animals attained their original weights.

Preparation and incubation of tissues

Adipose tissue. After killing the rats by decapitation, the epididymal fat pads were removed just distal to the major blood vessels. The pads were transferred into preweighed vessels containing isotonic saline, weighed, and cut into 10–30-mg pieces. The pieces were then decanted on gauze, adherent oil droplets were removed with saline wash, and amounts of tissue weighing about 500 mg were taken for the incubations.

The adipose tissue pieces were incubated at 37°C in 95% O_2–5% CO_2 with shaking (100 oscillations/min) in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 4% dialyzed bovine serum albumin (fraction V, Armour Pharmaceutical Co., Ltd., Eastbourne, England), 5 mmoles/l glucose, and additional substances as indicated separately for each experiment. After a preincubation of 10 min, the radioactive substrates, sodium [1-^{14}C]acetate (58 mCi/mmole), DL-[2-^{3}H]mevalonic acid lactone (500 mCi/mmole), D-[1-^{14}C]glucose (3 mCi/mmole), L-[U-^{14}C]leucine (311 mCi/mmole), or tritiated water (5 Ci/g) (all purchased from Radiochemical Centre, Amersham, England), were added in amounts indicated separately for each experiment. The incubation time was 3 hr, and each incubation was carried out in triplicate.

After incubation, the tissue pieces were washed with isotonic saline to remove the label adhering from the medium, and the fat cells were separated from the other cellular elements and connective tissue by treatment with collagenase (Worthington Biochemical Corp., Freehold, N.J). The isolated fat cells were separated by flotation at low speed and washed four times with buffer solution (21). No radioactive cholesterol could be detected in the medium after incubation of adipose tissue, and collagenase digestion left only 4% of tissue cholesterol radioactivity in the cell-free medium. Thus, under the conditions used, a negligible fraction of newly synthesized cholesterol was lost from the cells (fat cells or stromal vascular cells).

Preparation and incubation of liver slices. Immediately after the rats were killed, the livers were excised, placed in saline, and weighed. Liver slices 0.3 mm thick were cut with a Stadie-Riggs type tissue slicer. The first slice with liver capsule was discarded and the second was taken for the experiment. The slices were incubated for 2 hr under conditions similar to those used for the adipose tissue pieces but without addition of albumin or glucose. After a 10-min preincubation the isotopes were added, and after incubation the slices were washed to remove adherent label.

Preparation of fat cell fractions

Isolated fat cells were disrupted mechanically and centrifuged at a low rate to obtain the bulk lipid fraction and the infranate containing the subcellular organelles. The particulate fraction was obtained by centrifuging the infranate at 10^5 *g* for 30 min at 4°C (22).

Preparation of lipoproteins

The serum VLDL, LDL, and HDL were prepared with a preparative Spinco model L-50 ultracentrifuge using the densities of 1.006, 1.063, and 1.21 (1.23 for rat serum), respectively (23). The separated lipoproteins were dialyzed against Krebs-Ringer bicarbonate buffer and used within 2 days.

CHEMICAL METHODS

Isolation of digitonin-precipitable sterols

Lipids of fat cells or liver slices were extracted with chloroform–methanol 2:1, and 0.5% saline was added to separate the phases. The lower phase was equilibrated once with pure solvent upper phase containing salt to remove nonlipid contaminants (24). From the washed lipid extract one aliquot was taken for counting of radioactivity of total lipids and another for triglyceride determination (25). The remainder of the lipid extract was saponified with 1 N NaOH in 90% ethanol for 1 hr at 75°C. After saponification, the mixture was made up to a 50% ethanolic solution and extracted with petroleum ether to remove nonsaponifiable lipids.

The petroleum ether extract was taken to dryness, and the sterols were then isolated as their digitonides by the procedure of Sperry and Webb (26). The digitonides were dissolved in 1 ml of methanol and their ^{3}H and ^{14}C ac-

tivities were assayed (27). The recovery of added radioactive cholesterol was always more than 90%.

For purposes of fractionation of the digitonin-precipitable sterols into cholesterol and methyl sterols by TLC, the free sterols were regenerated by the method of Sperry (28). TLC was carried out on a 20 × 20 cm glass plate coated with a suspension of silica gel G (E. Merck, Darmstadt, Germany) in distilled water (60 g/120 ml) and activated for 1 hr at 100°C. Standards (lanosterol and cholesterol) and sterol mixture were applied, and the plates were developed in heptane–ethyl ether 47:53 (v/v). The plates were sprayed with a half-saturated aqueous solution of rhodamine 6G, and the sterols were detected under UV light. This TLC procedure offered a good separation of the cholesterol and methyl sterol fractions. Sterols were scraped off and eluted with diethyl ether, and aliquots were taken for determination of radioactivity and for GLC analysis of methyl sterols and cholesterol (29).

Determination of fat cell lipid content

For determination of cell number, size, and lipid content, one sample of adipose tissue was incubated with collagenase, and free fat cells were prepared as stated above. A homogeneous suspension of free fat cells in albumin–Krebs-Ringer bicarbonate buffer was obtained by mixing the suspension with a slowly running propeller. One aliquot was taken for cell lipid analysis and another for study of cellularity. Total cell lipids were extracted as described, and free and esterified cholesterol and triglycerides were separated on TLC with heptane–ethyl ether–glacial acetic acid 85:15:2. The losses of lipids were corrected by the recovery of added ^{14}C-labeled cholesterol, cholesteryl palmitate, and tripalmitin. To the free and esterified cholesterol fractions, 5α-cholestane was added as internal standard for GLC estimation (30). The triglycerides were measured as above.

Several 10-μl aliquots were taken from the fat cell suspension, and cells were counted in counting chambers with a depth of 0.2 mm. Fat cell size was measured from 500 cells, using a microscope with an ocular micrometer.

To get the average amount of lipid per cell, the lipid content per volume of the homogeneous cell suspension was divided by the number of cells in that volume. To get the radioactivity per cell, the radioactivity per unit weight of triglyceride was divided by the corresponding cell number, when the amount of triglyceride in the cell was known. To get the cell number per gram of adipose tissue, the triglyceride content per gram of tissue was divided by the triglyceride content of one fat cell.

Assay of radioactivity

All samples were counted in a Packard model 3003 Tri-Carb liquid scintillation counter at 5°C using 0.5% 2,5-diphenyloxazole (PPO) and 0.03% *p*-bis-[2-(5-phenyloxazolyl)]benzene (POPOP) in toluene. Absolute activity levels (dpm) were determined by means of an external standard.

Calculations

The data are expressed as moles of radioactive substrate incorporated into digitonin-precipitable sterols per unit weight of triglyceride or tissue per 1 hr of incubation. The number of moles of substrate incorporated was calculated by dividing the dpm of ^{3}H or ^{14}C in the digitonin-precipitable sterols by the specific activity of the substrate in the incubation medium. In order to correct for a loss of 33% of the radioactivity as $^{14}CO_2$ during sterol biosynthesis from [1-^{14}C]acetate and for a similar loss of 33% of ^{3}H from [2-^{3}H]mevalonate during the conversion of lanosterol to cholesterol (31), the incorporation rates of acetate and mevalonate were multiplied by 1.5. The incorporation rates of glucose were multiplied by a factor of 1.2 because [1-^{14}C]glucose gives rise to acetyl CoA labeled in position 2; and 3 of 18 labeled carbon atoms are lost during demethylation steps. When using [U-^{14}C]leucine as a precursor of cholesterol, the incorporation rates were multiplied by 1.71 to correct for the loss of labeled carbon atoms as CO_2 in three different steps of the conversion. The calculations are based on the assumption that all digitonin-precipitable sterol was cholesterol.

RESULTS

Distribution of free and esterified cholesterol within the fat cell

As in most other tissues, the cholesterol in fat cells was mainly in nonesterified form (**Table 1**). Of the total cholesterol in the whole fat cell, 10% was esterified; in the particulate fraction, only 3% was esterified. Averages of 77% of the cellular free cholesterol and 92% of the esterified cholesterol were present in the lipid droplet.

Sterol synthesis in adipose tissue cells

Upon incubation of adipose tissue with [1-^{14}C]acetate, the cholesterol was labeled. Of the total radioactivity pres-

TABLE 1. Amount of free and esterified cholesterol in rat adipocyte and its subfractions

	Adipocyte	Lipid Droplet	Particulate Fraction
		μg/g cell TG	
Free cholesterol	476 ± 23	367 ± 13 (77)	109 ± 11 (23)
Esterified cholesterol	54 ± 9	50 ± 9 (92)	4 ± 0.3 (8)
Total cholesterol	530 ± 19	417 ± 13 (79)	113 ± 11 (21)

Values are means ± SEM of three separate experiments in which cells from two rats were pooled. Numbers in parentheses are percentages of the value for the whole fat cell.

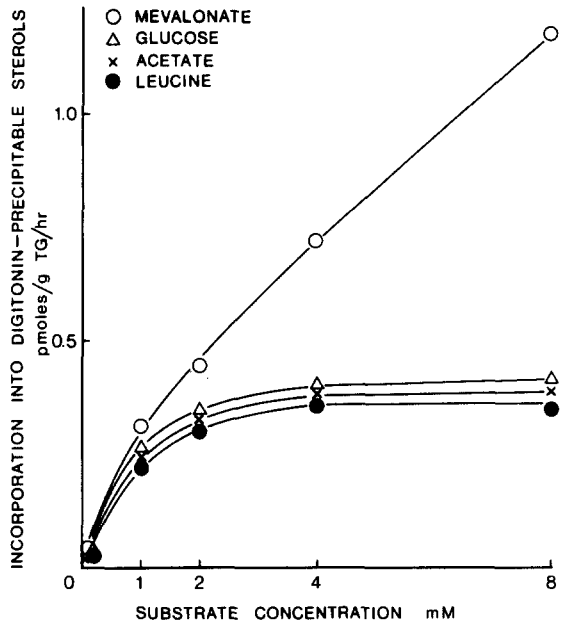

Fig. 1. Effect of substrate concentration on rate of incorporation of mevalonate, glucose, acetate, and leucine into fat cell cholesterol. Incubation medium: 4 ml of Krebs-Ringer bicarbonate buffer, 4 g/100 ml bovine serum albumin, and 5 mmoles/l of glucose (except in [^{14}C]glucose experiments). After a 10-min preincubation, DL-[2-^{3}H]mevalonate, D-[1-^{14}C]glucose, [1-^{14}C]acetate, or L-[U-^{14}C]leucine and corresponding unlabeled substrates were added to obtain the concentrations indicated. Incubation was for 3 hr at 37°C.

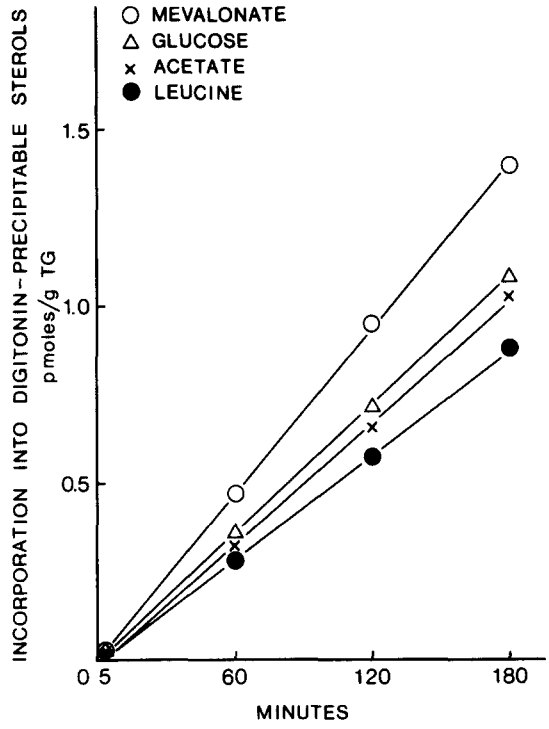

Fig. 2. Time course of incorporation of mevalonate, glucose, acetate, and leucine into fat cell cholesterol. Incubation medium and the labeled substrates are indicated in the legend to Fig. 1. The substrate concentration was 2.0 mM in each experiment.

ent in tissue cholesterol, an average of 44% was recovered in fat cells, 52% was present in the sediment containing stromal vascular cells, and negligible amounts remained in the medium. Thus, little of the newly synthesized cholesterol was lost by disruption of the cells during incubation, and this justified the use of a procedure in which fat cells were prepared after incubation of adipose tissue with radioactive precursors.

TABLE 2. Labeling of methyl sterols and of free and esterified cholesterol of fat cells upon incubation with radioactive acetate and mevalonate

Substrate	Product		
	Methyl Sterols	Cholesterol	
		Free	Esterified
[1-^{14}C]Acetate			
% Distribution	7.8	89.1 ± 4.4[a]	3.1 ± 0.5
Specific activity (dpm/μg)	11.5	0.46	0.14
DL-[2-^{3}H]Mevalonate			
% Distribution	19.7	70.1 ± 5.9	10.2 ± 2.3
Specific activity	22.3	0.27	0.35

Incubation mixture: 6 ml of Krebs-Ringer bicarbonate buffer, 5.0 mM glucose, 4 g/100 ml bovine serum albumin, 1.3 mM [1-^{14}C]acetate (0.55 mCi/mmole), and 3.5 nM DL-[2-^{3}H]mevalonate (500 mCi/mmole). Incubation was for 3 hr at 37°C.

[a] Means ± SEM (n = 3).

Sterol synthesis in fat cells

In addition to labeled acetate, the fat cells incorporated DL-[2-^{3}H]mevalonate, D-[1-^{14}C]glucose, L-[U-^{14}C]leucine (**Fig. 1**), and tritiated water into cholesterol upon in vitro incubation. Maximal rates of incorporation were achieved at an approximate concentration of 3.0 mM for each substrate with the exception of mevalonate. The approximate apparent K_m values for glucose, acetate, and leucine were 0.65, 0.70, and 0.75 × 10^{-3} M, respectively. The incorporation occurred at a linear rate for at least 3 hr (**Fig. 2**). Furthermore, the rate of incorporation was linearly correlated with the amount of tissue up to at least 1 g.

The distribution of the label from [1-^{14}C]acetate among different sterol fractions of fat cells and the specific activity of products appear in **Table 2.** The main part of sterol radioactivity was present in free cholesterol;[1] this agrees with previous studies that indicated that, with the exception of skin, testis, and thrombocytes, the rat tissues incorporate little acetate carbon into 3-β-hydroxysterols other than cholesterol (32, 33). The specific activity of methyl sterols was much higher than that of free or esterified cholesterol, thus demonstrating the rapid turnover of

[1] Digitonin-precipitable sterols are referred to as cholesterol in the text.

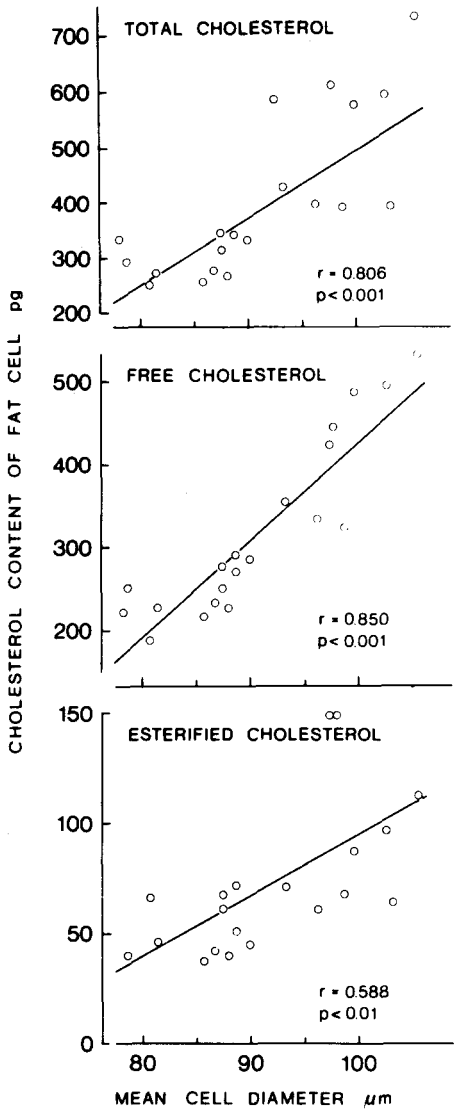

Fig. 3. Relationship between fat cell total, free, and esterified cholesterol content and cell diameter.

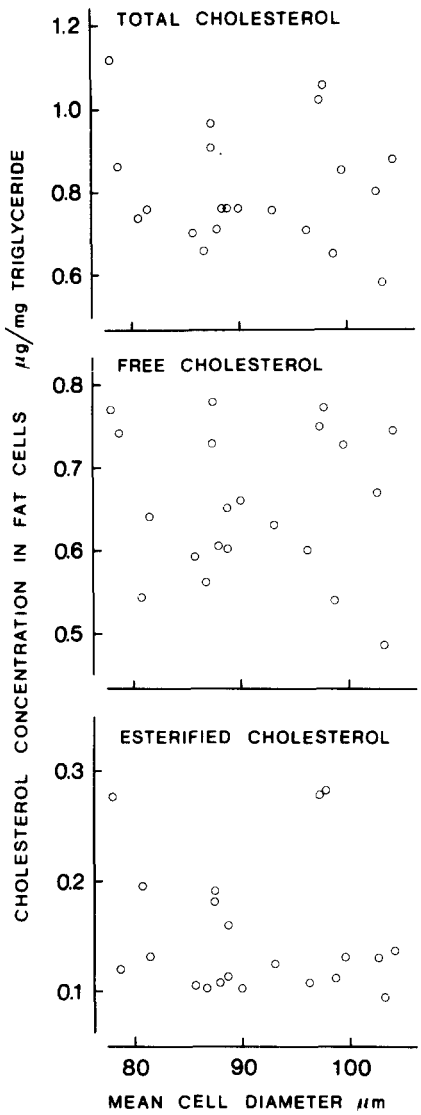

Fig. 4. Relationship between fat cell cholesterol/triglyceride mass ratio and cell size.

these intermediates. The esterified cholesterol, on the other hand, had a lower specific activity than the free fraction. Relatively more of the [2-^{3}H]mevalonate was recovered in the methyl sterol fraction compared with [1-^{14}C]acetate, and less was recovered in free cholesterol (Table 2).

Cholesterol concentration and synthesis in relation to fat cell size

Populations of fat cells with mean diameters ranging from 78 to 105 μm were prepared from rats 23–25 wk old with different degrees of adiposity. Some variation in the body fat and in fat cell size was spontaneous whereas some was induced by overfeeding some of the animals with a high-sucrose diet (see Materials and Methods). This diet did not influence cholesterol synthesis in fat cells of equal size and, therefore, the results from animals fed the two different diets were pooled.

The mean fat cell diameter was positively correlated with body weight ($r = +0.62$, $P < 0.001$). The amount of fat cell cholesterol increased in close correlation with the cell diameter (**Fig. 3**). The degree of this association was highest for total and free cholesterol and somewhat less for esterified cholesterol. An increase of mean cell diameter from 80 to 100 μm caused a doubling of cell cholesterol content corresponding to the approximate increase of cell volume. Because the fat cell triglyceride content was highly correlated with fat cell size ($r = 0.95$), the cholesterol/triglyceride ratio remained constant in cells of different sizes (**Fig. 4**). The rate of cholesterol synthesis from acetate in relation to fat cell size is shown in **Fig. 5.** The amount of cholesterol synthesized per cell was directly re-

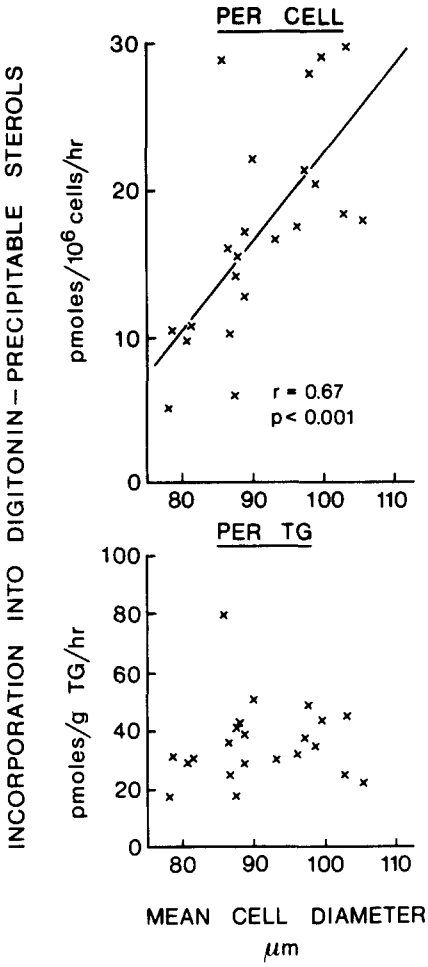

Fig. 5. Fat cell cholesterol synthesis from acetate in relation to cell size. The upper panel shows the rate of cholesterol synthesis per cell, the lower panel the rate per unit mass of cell triglyceride. Incubation medium as in Fig. 2, incubation time 3 hr.

lated to cell size. An increase of cell diameter from 80 to 100 μm resulted in a three- to fourfold enhancement of the rate of cholesterol synthesis. On the other hand, cholesterol synthesis expressed per cell triglyceride content remained constant and independent of cell size. Therefore, in subsequent studies the synthesis rates have been expressed on this basis (triglyceride content) in order to eliminate the influence of variable cell size.

Relative rates of cholesterol synthesis in adipose tissue and liver

In order to compare the rates of synthesis of cholesterol in vitro in adipose tissue and in liver, the incorporation of [1-^{14}C]acetate into cholesterol was determined in liver slices, in pieces of adipose tissue, and in fat cells isolated from adipose tissue after incubation. The results (**Table 3**) show that, expressed per unit of tissue weight, the rate of cholesterol synthesis in adipose tissue was only 2% of the corresponding value in the liver. Assuming that epididymal fat is representative of adipose tissue at other sites, and taking 8% of body weight as the adipose tissue mass, one can estimate that whole body adipose tissue cholesterol synthesis is 4% of that in the liver.

Effect of fasting and refeeding on fat cell cholesterol content and synthesis

Although it is well established that the triglyceride content of adipocytes is profoundly influenced by the calorie balance, no studies have been made to determine whether the fat cell cholesterol is also depleted during starvation and restored during refeeding. In order to examine the effect of nutritional balance on the fat cell cholesterol content, rats were fasted for 60 hr or were fasted and then refed for 72 hr. Fasting caused an average decrease of fat cell triglyceride to 41% of the prefasting value whereas refeeding increased it to 76% of the level found in nonfasted control animals (**Fig. 6,** lower panel). Free cholesterol followed closely the cell triglyceride depletion and accumulation so that the free cholesterol concentration in the fat cell remained unchanged during fasting and refeeding (Fig. 6, upper panel). On the other hand, the esterified cholesterol content of the fat cell remained constant during the changes of calorie balance, which means that the concentration of cholesteryl ester in adipocytes was in-

TABLE 3. Rates of acetate conversion into liver and adipose tissue cholesterol of fed rats

Substrate	Per Unit Weight		In Whole Organ	
	Liver	Adipose Tissue	Liver	Adipose Tissue
	nmoles/g wet wt/hr	*nmoles/g TG/hr*	*nmoles/organ/hr*	
[1-^{14}]Acetate	26.40 ± 2.25[a]	0.53 ± 0.03 (2.0)[b]	342 ± 30	14.4 ± 0.8 (4.2)[b]

Incubation mixture: 6 ml of Krebs-Ringer bicarbonate buffer, 5 mM glucose and 4 g/100 ml bovine serum albumin (both omitted from incubations with liver slices), and 1.3 mM [1-^{14}C]-acetate (0.55 mCi/mmole). Incubation was for 3 hr at 37°C. Fat cells were isolated after incubation.

[a] Means ± SEM from three rats.

[b] Percentage of corresponding value for liver.

creased during fasting and reduced during the subsequent refeeding period (Fig. 6).

In contrast to lipogenesis, cholesterol synthesis in many extrahepatic tissues is not influenced by the nutritional state or calorie balance of the animal (17). No data have so far been reported on the possible control of cholesterol synthesis in adipose tissue by these factors, even though the extreme sensitivity of fat cell glyceride synthesis to fasting and feeding is well known. The effect of a 72-hr fasting period on cholesterol synthesis in fat cells compared with that in liver is shown in **Table 4.** Tritiated water was used as a precursor in addition to acetate and mevalonate in order to avoid misinterpretation of the incorporation data because of a possible change of intermediate pool sizes as a result of fasting. Fasting suppressed the incorporation of each substrate into fat cell cholesterol. The response of fat cell cholesterologenesis to prolonged fasting was quantitatively similar to that of the liver. In both tissues the incorporation of acetate carbon was suppressed more than that of tritiated water; this difference might be due to enlargement of the cellular acetate pool during fasting.

In order to estimate the extent to which the observed changes in the incorporation of labeled acetate reflected only dilution of this precursor in the intracellular acetyl CoA pool, the flux of acetate into cholesterol was calculated from ^3H_2O incorporation by multiplying the latter by 1.31 (34). These values appear in Table 4, which shows that in the livers of fed rats 38.6% of the acetate incorporated into cholesterol originated from endogenous sources ("dilution"). In the livers of fasted rats the corresponding figure was 96.0%, indicating that only 4.0% of the acetate incorporated into cholesterol was derived from the medium. In fat cells of fed and fasted rats, the corresponding percentages were 58.8% and 93.7%, respectively. Thus, in both tissues, fasting greatly increased the contribution of endogenous acetate in cholesterol synthesis. The corrected acetate incorporation data show that fasting suppressed cholesterol synthesis to a similar extent in liver and adipose tissue. Our result for fasting liver is very similar to that recently reported by Dietschy and Brown (35).

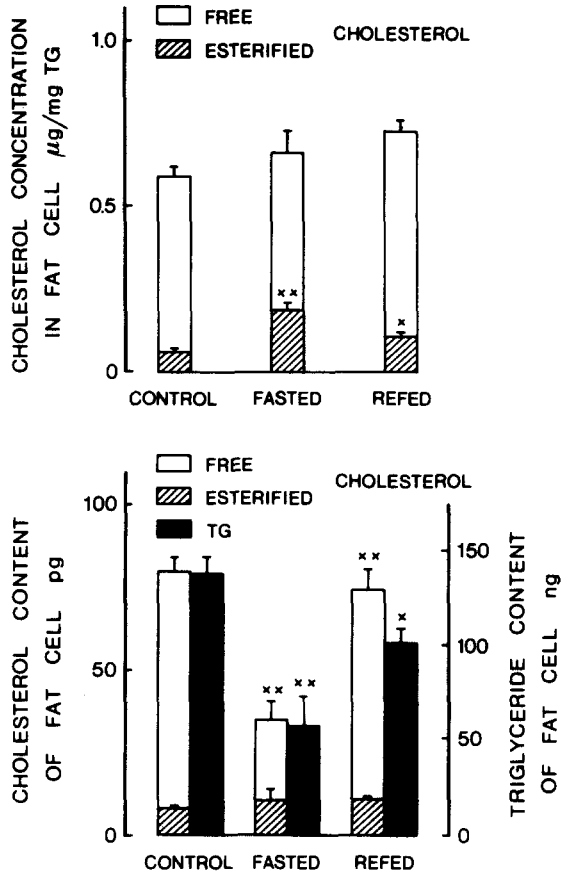

Fig. 6. Effect of fasting (60 hr) and of refeeding (72 hr) on cholesterol content and relative cholesterol concentration (cholesterol/triglyceride mass ratio) of fat cells. Differences were tested between fasted and control rats and between refed and fasted rats (×, $P < 0.05$; ××, $P < 0.01$).

Upon refeeding, the fasted animals increased their fat cell cholesterol synthesis from acetate 40-fold, a rate that

TABLE 4. Effect of fasting on substrate incorporation into cholesterol in liver and in fat cells

Nutritional State	Liver Slices			Fat Cells		
	[1-^{14}C]Acetate	DL-[2-^{3}H]-Mevalonate	^3H_2O	[1-^{14}C]Acetate	DL-[2-^{3}H]-Mevalonate	^3H_2O
	nmoles/g wet wt/hr			*pmoles/g TG/hr*		
Fed	23.4 ± 3.0[a] (38.1)[b]	119.3 ± 22.5	29.1 ± 9.0	387.1 ± 17.1 (939.3)[b]	741.3 ± 38.1	717.0 ± 34.5
Fasted 72 hr	0.2 ± 0.0 (5.2)[b]	2.0 ± 0.9	4.0 ± 0.8	7.5 ± 3.0 (118.5)[b]	165.5 ± 15.5	89.8 ± 1.1
	0.8[c]	1.7[c]	13.7[c]	1.9[c]	22.3[c]	12.5[c]

Basal incubation medium as described in Table 3. Radioactive precursors: 2 mM [1-^{14}C]acetate (0.5 mCi/mmole), 2 mM DL-[2-^{3}H]-mevalonate (1.0 mCi/mmole), or 0.4 Ci of ^3H_2O. Fat cells were isolated after incubation.

[a] Means ± SEM (n = 3).

[b] Numbers in parentheses indicate the flux of C_2 units into cholesterol calculated from the incorporation of ^3H_2O (^3H_2O multiplied by 1.31).

[c] Percentage of the value for fed animal.

TABLE 5. Effect of in vitro addition of glucose and insulin on incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols of fat cells from fed, fasted, and fasted–refed rats

Nutritional State	Additions[a]		Incorporation into Fat Cell Digitonin-precipitable Sterols
	Glucose	Insulin	
	mM	200 *μU/ml*	*% of value for fed animal*
Fed	5	−	100
	5	+	103 ± 6[b]
	15	+	106 ± 4
Fasted for 48 hr	5	−	23 ± 6
	5	+	21 ± 3
Fasted for 72 hr	−	−	6 ± 0
	−	+	6 ± 1
	5	+	6 ± 2
	15	−	11 ± 4
	15	+	11 ± 3
Fasted for 48 hr and refed for 48 hr	5	−	256 ± 25
	5	+	267 ± 17

[a] Pieces of adipose tissue were incubated in 6 ml of Krebs-Ringer bicarbonate buffer containing 4 g/100 ml bovine serum albumin and 1.3 mM [1-^{14}C]acetate (0.55 mCi/mmole). Incubation was for 3 hr at 37°C. After incubation the fat cells were isolated as described in Materials and Methods.

[b] Means ± SEM (n = 3).

was about 2.5 times higher than that found in normally fed animals (**Table 5**).

Effect of glucose and insulin on cholesterol synthesis in fat cells

Addition of glucose and insulin to the incubation medium did not influence the incorporation of acetate into cholesterol of fat cells taken from normally fed, fasted, or fasted–refed rats (Table 5). On the other hand, insulin stimulated the incorporation of both [1-^{14}C]glucose and L-[U-^{14}C]leucine into fat cell cholesterol (**Table 6**). This effect most probably is secondary to the enhanced transport of both substrates into the cell.

TABLE 6. Effect of in vitro addition of insulin on glucose and leucine incorporation into digitonin-precipitable sterols of fat cells

Additions	Incorporation into Fat Cell Digitonin-precipitable Sterols	
	[1-^{14}C]Glucose	L-[U-^{14}C]Leucine
	%	
None	100	100
Insulin, 200 μU/ml	180	244

Incubation mixture: 6 ml of Krebs-Ringer bicarbonate buffer, 4 g/100 ml bovine serum albumin, 5 mM D-[1-^{14}C]glucose (0.05 mCi/mmole), and 2 mM L-[U-^{14}C]leucine (0.42 mCi/mmole). Incubation was for 3 hr at 37°C.

Effect of stimulated lipolysis on fat cell cholesterologenesis

Stimulation of intracellular lipolysis with theophylline and with dibutyryl cyclic AMP was accompanied by marked inhibition of cholesterol synthesis from acetate, mevalonate, and tritiated water (**Table 7**). The degree of inhibition was related to the activity of lipolysis (release of FFA) and was approximately similar for both compounds. The incorporation of tritiated water was inhibited less than that of acetate and mevalonate, a difference probably accounted for by the increase in the intracellular pool size of the two intermediates during enhanced lipolysis.

Effect of cholesterol and cholestyramine feeding on fat cell cholesterol synthesis

Because hepatic cholesterol synthesis is suppressed by dietary cholesterol and stimulated by bile acid-sequestering agents, it seemed advisable to study the effect of these factors also on sterol synthesis in adipose tissue. Simultaneous assays of liver and fat cell cholesterol synthesis were carried out in rats fed cholesterol or cholestyramine for 2 wk. The results are shown in **Fig. 7**. Neither treatment influenced the fat cell cholesterol content. The serum cho-

TABLE 7. Effects of theophylline and dibutyryl cyclic AMP on substrate incorporation into fat cell cholesterol

Additions		Incorporation into Fat Cell Digitonin-precipitable Sterols			
		[1-^{14}C]Acetate	DL-[2-^{3}H]-Mevalonate	$^{3}H_2O$	FFA Release
	mM	%	%	%	*μmoles/g TG/hr*
Theophylline	0	100 (277)[a]	100 (468)[a]	100 (693)[a]	11.0
	1.0	54	70	77	33.2
	2.0	27	32	53	57.2
	3.0	16	17	38	81.6
Dibutyryl cyclic AMP	0	100	100	100	10.6
	1.0	92	58	97	23.2
	2.0	42	18	56	69.4
	3.0	11	14	37	103.0

Incubation mixture: 8 ml of Krebs-Ringer bicarbonate buffer, 5.0 mM glucose, 640 mg of bovine serum albumin, and 2.0 mM [1-^{14}C]acetate (0.5 mCi/mmole), 2.0 mM DL-[2-^{3}H]mevalonate (1.0 mCi/mmole), or 0.5 Ci of $^{3}H_2O$ and the additions indicated. Incubation was for 3 hr at 37°C.

[a] Basal incorporation rate (100%), pmoles/g TG/hr.

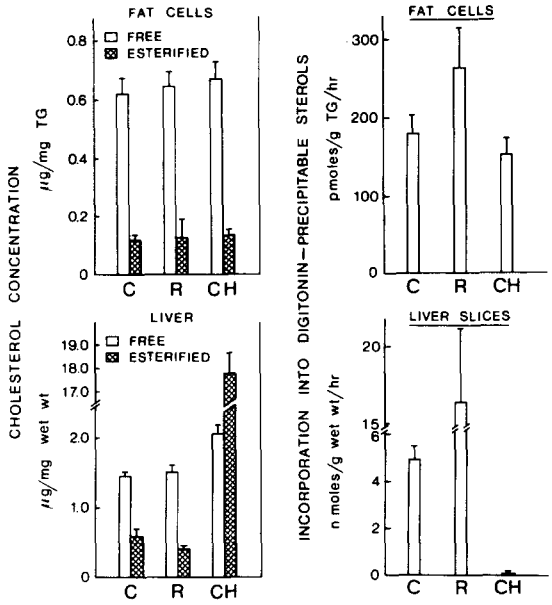

Fig. 7. Effect of cholestyramine (R) and high cholesterol diet (CH) on cholesterol concentration (left panels) and rate of synthesis (right panels) in fat cells and liver. C, control rats receiving basal chow. Cholesterol synthesis was determined with [1-^{14}C]acetate. Incubation conditions as in Fig. 2, incubation time 3 hr. None of the differences between controls and treatment groups is significant at $P = 0.05$.

lesterol level rose with cholesterol feeding from an average of 104 ± 5 to 212 ± 39 mg/100 ml ($P < 0.001$), and the liver cholesteryl ester content increased 30-fold. Cholestyramine-fed animals showed no significant change in liver or serum cholesterol levels. The rate of incorporation of acetate into fat cell cholesterol was not significantly affected by either treatment, whereas liver cholesterol synthesis showed the typical responses.

Effect of serum lipoproteins on fat cell cholesterol synthesis

The addition of different rat or human serum lipoproteins to the incubation medium produced variable effects on fat cell cholesterol synthesis from acetate and mevalonate (**Table 8**). The VLDL of both species significantly inhibited the incorporation of acetate into cell cholesterol already at a cholesterol concentration of 40 μg/ml, whereas the LDL was inhibitory only at much higher concentrations and HDL did not inhibit the acetate incorporation at all. Preincubation of fat cells with rat serum VLDL for 2 hr before addition of the labeled substrates did not significantly alter the synthesis of cholesterol from that seen in a medium where VLDL was added simultaneously with the precursor. Upon incubation of fat cells with lipoprotein-free serum (bottom fraction of centrifugation at 1.21), the incorporation of both acetate and mevalonate into cholesterol was stimulated compared with the incubation without any serum. No newly synthesized cholesterol was released into the medium in these incubations.

TABLE 8. Effect of serum lipoproteins and nonlipoprotein serum on incorporation of [1-^{14}C]acetate and DL-[2-^{3}H]-mevalonate into adipocyte cholesterol

Fraction	Cholesterol in Medium	Incorporation into Cholesterol: [1-^{14}C]-Acetate	Incorporation into Cholesterol: DL-[2-^{3}H]-Mevalonate
	μg/ml	%	%
Buffer	0	100	100
Rat			
VLDL	40	85	100
	90	65	92
LDL	20	103	110
HDL	20	98	100
Nonlipoprotein serum	0	153	198
Human			
VLDL	40	75	92
	200	33	49
LDL	70	97	108
	200	75	93
	500	70	110
HDL	20	104	105
	200	96	
Nonlipoprotein serum	0	188	136

Free fat cells were incubated in 4 ml of Krebs-Ringer bicarbonate buffer containing 5 mM glucose, 4 g/100 ml bovine serum albumin, 2 mM [1-^{14}C]acetate (6.25 mCi/mmole), 2 mM DL-[2-^{3}H]mevalonate (6.25 mCi/mmole), and the additions indicated. Incubation was at 37°C for 3 hr. The lipoproteins or nonlipoprotein serum was added 10 min before the labeled substrates.

DISCUSSION

In agreement with the results recently reported by Farkas, Angel, and Avigan (1), we found that cholesterol is a constant structural constituent of white fat cells and it is mainly located in the fat droplet. The finding that the cholesterol/triglyceride ratio is fairly constant and largely independent of fat cell size suggests that the deposition and mobilization of both lipids occurs simultaneously and in a given stoichiometric ratio. This constancy of the cholesterol concentration, which is far below any maximum solubility (36), in the stored fat favors the view that cholesterol is required for stabilization of the structure of the fat droplet or that it has a role in the action of lipase on the surface of the stored triglyceride mass. On acute changes of the fat cell triglyceride by fasting–refeeding, the free cholesterol closely follows the fluctuations of cellular triglyceride, but esterified cholesterol content remains unchanged. It is possible that, contrary to free cholesterol, the cholesteryl esters are not freely exchangeable with plasma lipoproteins and they must undergo hydrolysis before being released from the cell. Evidence for inability of cholesteryl esters to pass the plasma membrane has been recently obtained in ascites cells (37), and we have observed that esterified cholesterol of serum lipoproteins is not in-

corporated into rat fat cells during in vitro incubation.[2] Cholesteryl esterase is known to be present in adipose tissue (38), but perhaps it is not activated by fasting to the same extent as triglyceride lipase.

In the present study, feeding a high cholesterol diet to rats caused a significant increase in serum and liver cholesterol. In spite of these changes, the fat cell cholesterol content remained constant. This finding is in accordance with previous observations of Ho and Taylor (2), but it is opposite to the results recently reported by Angel and Farkas (39), who found a decrease in serum cholesterol and an increase in fat cell cholesterol concentration upon feeding rats a high cholesterol diet. This discrepancy might be accounted for by the difference in the quality of fat added to the high cholesterol diet. We used olive oil whereas Angel and Farkas (39) used corn oil. The increase in fat cell cholesterol from feeding a corn oil–high cholesterol diet might be due to redistribution of cholesterol between plasma and tissue pools during feeding of polyunsaturated fats (40).

Like all other tissues studied so far, the fat cells are able to synthesize cholesterol from acetate and mevalonate. Under our incubation conditions, the fat cells converted acetate to cholesterol at an average rate of 0.9 nmole/g/hr when corrected for dilution by endogenous acetate. This rate is only 2% of that obtained in the liver, and in this respect our results agree with the findings of Dietschy and Wilson (17), who studied cholesterol synthesis in squirrel monkey, even though the absolute rates of cholesterol synthesis in both tissues are lower in our rats. Calculated per whole organ, the in vitro cholesterol synthesis in adipose tissue amounts to about 4% of that in the liver. Thus, the contribution of the total adipose tissue to body cholesterol turnover remains small. However, it is not known whether cholesterol synthesis in adipose and other extrahepatic tissues is a significant factor in determining the serum cholesterol level.

In contrast to other peripheral tissues (32), cholesterol biosynthesis in the fat cell was found to be markedly influenced by caloric intake. Fasting was accompanied by a strong suppression of cholesterologenesis whereas refeeding caused an overshoot of cholesterol synthesis up to three times above the control values. In this respect the regulation of cholesterol formation in fat cells is similar to that in the liver (17, 32), even though less remarkable effects of refeeding on hepatic cholesterologenesis have been reported (41). With the possible exception of intestine (42), other extrahepatic tissues continue to synthesize cholesterol during fasting at a rate that is similar to or only slightly lower than that occurring during the fed state (32).

This type of regulation of cholesterol synthesis in fat cells seems physiologically meaningful because cholesterol is mobilized with triglyceride during fasting and reaccumulates during positive calorie balance. Calculation of the synthesis rate from the in vitro data and the extrapolation of these data to in vivo conditions shows that a complete cessation of synthesis does not account for the observed drop in fat cell cholesterol content during fasting, nor does the increase in the synthetic rate during refeeding correspond to the rapid net increase in adipocyte cholesterol mass. Thus, in addition to the appropriate changes in sterol synthesis according to calorie balance, an active net release and uptake of fat cell cholesterol must occur during fat mobilization and deposition, respectively. Exchange of labeled cholesterol between fat cells and serum lipoproteins occurs in both directions.

The mechanism by which sterol synthesis in fat cells is mediated by calorie balance is not clear. One highly plausible possibility is suppression and activation of HMG CoA reductase synthesis during fasting and refeeding, respectively. Like the regulation of many other enzymes of adipose tissue, this might be performed by insulin and glucose controlling the synthesis of new enzyme protein. Insulin has been shown to increase the activity of HMG CoA reductase in rat liver (43) and in cultured human fibroblasts (44), and insulin concentration in plasma is low during fasting and increases on refeeding. Another possibility is that cholesterol synthesis is regulated by the cellular FFA concentration. The present study showed that upon stimulation of lipolysis by either theophylline or dibutyryl cyclic AMP cholesterologenesis in fat cells, was markedly suppressed. From these results it cannot be determined, however, whether the inhibitory factor is cellular FFA or cyclic AMP, because both are increased in a parallel manner by the two agents. In the liver, cyclic AMP inhibits cholesterol synthesis (45, 46) evidently by reducing the activity of HMG CoA reductase (46), and a similar mechanism could well operate in adipose tissue. On the other hand, the significance of physiological concentrations of cyclic AMP in the regulation of hepatic cholesterol synthesis has been challenged (47). In our experiments, a striking suppression of fat cell cholesterol synthesis could be obtained by increasing the endogenous cyclic AMP concentration with theophylline, i.e., by conditions that are close to physiological.

FFA may also influence cholesterol synthesis. In perfused rat liver, high concentrations of FFA have recently been found to stimulate cholesterol synthesis and secretion into the medium (48), but, in isolated liver cells, addition of FFA to the medium reduces cholesterol synthesis (49). It should be noted, however, that under all conditions the intracellular FFA concentration is much less in the liver than in adipose tissue. The fluctuation of cholesterol syn-

[2] Kovanen, P. T., and E. A. Nikkilä. To be published.

thesis in fat cells with fasting–refeeding could thus be primarily accounted for by changes in the plasma insulin concentration and, secondly, by cyclic AMP and FFA content of the cell.

In contrast to the situation in liver, cholesterol synthesis in fat cells was not sensitive to variations in dietary cholesterol. As the synthesis was studied only in vitro in a cholesterol-free medium, it is still possible that some depression of cholesterologenesis was present in vivo in the presence of an elevated serum cholesterol concentration. The feedback inhibition of cholesterol biosynthesis obviously implies an increased concentration of cholesterol at the site of synthesis; in the rat, a significant accumulation of excess dietary cholesterol occurs only in the liver (2). In other species, the cholesterol content of adipose tissue can be increased by feeding a high cholesterol diet (2), and then some inhibition of cholesterol formation may be observed (17).

The rate of cholesterol synthesis in fat cells was found to increase in close correlation with cell size. The mechanism of the stimulation of sterol synthesis on cell enlargement remains obscure because the increase in fat cell size is limited to accumulation of bulk fat without a concomitant augmentation of cellular protein (50) or cytoplasmic mass (51). The large fat cells are also more resistant to insulin action than small ones (50). However, the glucose incorporation into fat cell triglyceride has also been shown to increase in a rough correlation with cell size (8, 50).

As obesity is associated with markedly increased cholesterol production, the rate of which is positively correlated with the amount of body fat (14–16), it has been suggested that adipose tissue might be the source of extra cholesterol synthesis in obesity (14). Nestel, Whyte, and Goodman (14) and Miettinen (15) have calculated that the increase in cholesterol formation in man amounts to 20 mg/day/kg of excess adipose tissue. Estimated from the present in vitro studies, the cholesterol production rate would be only 0.81 mg/kg of rat adipose tissue per 24 hr. The corresponding figure for monkey adipose tissue calculated from the data of Dietschy and Wilson (17) is 1.7 mg/kg/day. Rates of similar order of magnitude have been observed for cholesterol synthesis in human adipose tissue.[2] It is thus evident that local cholesterol synthesis in adipose tissue hardly accounts for the increase in body cholesterol production in obesity. It is suggested that obesity stimulates cholesterol synthesis mainly in the liver (52), and this may possibly be accounted for by the increased flux of FFA and the hyperinsulinism (43, 44, 48, 53) often associated with obesity.

Fat cell cholesterol synthesis was found to be slightly inhibited by the presence of serum VLDL or LDL in the medium. A similar regulatory feedback mechanism has been recently found to operate in cultured human fibroblasts (54), which actually are closely related to adipocytes. In fat cells, LDL was far less effective than in fibroblasts, however, even though the experimental conditions in the two instances were not comparable. It might be thought that VLDL is a more physiological feedback inhibitor of cholesterol synthesis in fat cells than is LDL because the former lipoprotein is broken down in adipose tissue. Chylomicrons can suppress hepatic cholesterologenesis in rats in vivo (55), but it is not known whether the inhibitory particle is the original chylomicron or its "remnant" particle. The stimulation of cholesterol synthesis in fat cells by lipoprotein-free serum is a highly interesting phenomenon, particularly because a similar observation has been made in cultured fibroblasts (54).

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