Regulation of hepatic secretion of very low density lipoprotein by dietary cholesterol

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Abstract Male rats were fed a cholesterol-free diet or the same diet supplemented with either 0.05, 0.1, 0.25, 0.5, 1, or 2% C for 21 days to investigate the effects of cholesterol on secretion of very low density lipoprotein (VLDL). Cholesterol feeding increased plasma and hepatic concentrations of triglyceride (TG) and cholesteryl esters (CE) in a dose-dependent manner. Plasma VLDL and low density lipoprotein (LDL) lipids were elevated by cholesterol feeding, while the high density lipoprotein (HDL) lipids were reduced. The secretion of the VLDL by perfused livers from these cholesterol-fed rats was examined to establish the relationship between the accumulation of lipids in the liver and the concurrent hyperlipemia. Liver perfusions were carried out for 4 h with a medium containing bovine serum albumin (3% w/v), glucose (0.1% w/v), bovine erythrocytes (30% v/v), and a 10-mCi ^3H_2O initial pulse. Oleic acid was infused to maintain a concentration of 0.6 mM. Hepatic secretion of VLDL-TG, PL (phospholipid), free cholesterol (FC), and CE increased in proportion to dietary cholesterol and was maximal at 0.5% cholesterol in these experiments in which TG synthesis was stimulated by oleic acid. Secretion of VLDL protein and apoB by the perfused liver was also increased. The molar ratios of surface (sum of PL and cholesterol) to core (sum of TG and CE) lipid components of the secreted VLDL, regardless of cholesterol feeding, were the same, as were the mean diameters of the secreted particles. The molar ratios of surface to core lipid of VLDL isolated from the plasma also were not affected by cholesterol feeding. During perfusion with oleic acid of livers from the rats fed the higher levels of cholesterol, the hepatic concentration of CE decreased, while the level of TG was not changed. We conclude that the hypercholesterolemia and hypertriglyceridemia that occur in vivo from cholesterol feeding, concurrent with accumulation of CE and TG in the liver, must result, in part, from increased hepatic secretion of all VLDL lipids and apoB. The VLDL particles produced by the liver of the cholesterol-fed rat are assembled without modification of the surface lipid ratios (PL/FC), but contain a greater proportion of cholesteryl esters compared to triglyceride in the core, because of the stimulated transport of CE from the expanded pool in the liver. Therefore, the increase in transport of CE in VLDL reflects the formation of additional, rather than larger particles to accommodate the increased secretion of the expanded CE pool; this process appears to require additional TG, and it is probable that TG synthesis is stimulated by dietary cholesterol. Clearly, fatty acids and cholesterol, or both, can stimulate VLDL formation in the liver, although the composition of the VLDL will vary depending on the particular primary stimulus.—**Fungwe, T. V., L. Cagen, H. G. Wilcox, and M. Heimberg.** Regulation of hepatic secretion of very low density lipoprotein by dietary cholesterol. *J. Lipid Res.* 1992. **33**:179–191.

Supplementary key words triglyceride • apolipoprotein B • phospholipid • plasma lipoproteins • VLDL • HDL

It is well established that the liver regulates plasma levels of cholesterol and triglyceride (TG) by secretion and transport of these lipids in the VLDL and by removal of lipoproteins by receptor-mediated endocytosis, and that changes in nutritional and hormonal status alter the rate of assembly and secretion of VLDL particles (1–3). Although the primary stimulus for formation and secretion of the VLDL is the availability of free fatty acids (FFA) for TG synthesis, it was postulated that the ability of the liver to synthesize and secrete VLDL may also be regulated by availability of lipids or apolipoproteins that comprise the surface of the VLDL (4). In studies designed to evaluate the role of cholesterol in VLDL formation and secretion, Khan, Wilcox, and Heimberg (5) demonstrated that VLDL secretion was diminished when lovastatin was given to rats to reduce a putative hepatic metabolic pool of cholesterol. When cholesterol was included in the diet or when human LDL was added to the medium perfusing livers from the lovastatin-treated animals, VLDL secretion was returned toward control levels, suggesting the availability of cholesterol is required to form the VLDL and transport TG (and other lipids). Similar conclusions were subsequently derived from studies in intact rats (6). Inhibition of secretion of the VLDL, in fact, can be a major mechanism for induction of hepatic steatosis when a supply of FFA is available to the liver (4). Inhibition of the synthesis of

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; PL, phospholipid; TG, triglyceride; CE, cholesteryl ester; FFA, free fatty acid; TLC, thin-layer chromatography; AcAc-CoA, acetoacetyl-CoA; HMG, 3-hydroxy-3-methylglutaryl, FC free cholesterol.

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apolipoproteins (4, 7) or PL (8–10), essential components of VLDL, have been shown to diminish VLDL secretion and cause accumulation of TG in the liver. We have also observed that inhibition of cholesterol synthesis following lovastatin treatment reduced hepatic CE, but led to accumulation of TG in the liver (5). The effects were reversible on provision of a source of cholesterol. If availability of cholesterol (from synthesis or exogenous supplies) is rate-limiting for VLDL formation, it is possible that hepatic CE stores may be able to supply cholesterol for VLDL formation. Furthermore, when hepatic CE concentration is increased, as when cholesterol is fed, secretion of VLDL may be stimulated. To examine this possible stimulatory effect of increased levels of free cholesterol (FC) and/or CE in the liver on VLDL formation and secretion, male rats were fed a diet supplemented with varying concentrations of cholesterol for 3 weeks. Hepatic secretion of VLDL by perfused livers isolated from these animals was measured. The results of these studies indicate that cholesterol feeding stimulates hepatic accumulation of TG and CE, with only modest increases of FC, and that outward transport of CE and TG is enhanced by formation and secretion of additional VLDL particles. A preliminary report of this work has appeared (11).

METHODS

Materials and reagents

Male Sprague-Dawley rats, (100–125 g) were purchased from Harlan Labs (Indianapolis, IN). Cholesterol (USP), cholesterol-free diet, ^3H_2O, and Ecolume were purchased from ICN Biochemicals, Inc. (Costa Mesa, CA). Mazola corn oil (no cholesterol, 100% pure, food grade) was obtained locally. Oleic acid and bovine serum albumin (BSA, fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO) and the BSA was delipidated and purified before use as described previously (12). Silica-gel G thin-layer plates were purchased from Analtech Inc. (Newark, DE); DL-[3-^{14}C]HMG-CoA, D-[5-^{3}H]mevalonate, and [1-^{14}C]acetyl-CoA were purchased from New England Nuclear (Wilimington, DE). All other chemicals and reagents were analytical grade.

Animals and diets

Normal male Sprague-Dawley rats were maintained ad libitum on water and the basal diet, supplemented with corn oil to constitute 5% of the diet. The basal diet was a fat- and cholesterol-free, premixed semi-synthetic diet of the following composition: vitamin free casein, 25.0%; sucrose, 65.0%; alphacel, 6.0%; mineral and vitamin mixture, 4.0%. The animals were housed under a normal light–dark cycle, with lights on from 0700 to 1900. After 1 week on the basal diet, the animals were randomly distributed into seven dietary groups and fed either the same diet or the diet supplemented with 0.05, 0.1, 0.25, 0.5, 1.0 or 2.0% cholesterol (w/w). The cholesterol was dissolved in the corn oil and mechanically mixed in the diet. Cholesterol and corn oil were mixed thoroughly in the diet weekly and the diet mixes were stored at 4°C. The animals were fed these diets for 21 additional days. Rats were in a fed state at the initiation of the experiments.

Blood and liver samples

On the morning of the experiments, the rats were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). A surgical incision was made, and the animals were exsanguinated from the abdominal aorta. The livers were flushed with cold 0.9% NaCl, removed, and weighed. Samples were taken for lipid analysis and for enzyme assays.

Four ml of 0.9% NaCl was layered over 4 ml of plasma isolated from the blood (2 mg EDTA/ml) from individual animals in 9-ml polycarbonate centrifuge bottles and centrifuged in a Beckman 40 rotor at 12000 rpm for 30 min at 13°C. The floating chylomicron fractions were removed (0.5 ml) (5, 12).

Isolation of plasma lipoproteins

The plasma lipoproteins were separated by sequential ultracentrifugation (13), with modifications (14). The fractions at $d < 1.006$ g/ml (VLDL), d 1.006–1.063 g/ml (LDL), and d 1.063–1.210 g/ml ($HDL_2 + HDL_3$) were isolated using and L-80 ultracentrifuge equipped with 40 or 80 Ti rotor (Beckman Instruments, Fullerton, CA). Lipoprotein fractions were dialyzed for 48 h in Spectapore #1 tubing (Spectrum Medical Industries, Los Angeles, CA). Protein and lipid content (TG, PL, FC, and CE) were assayed in each lipoprotein fraction.

Liver perfusion studies

On the morning of the experiments, the rats were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). After removal of a blood sample from the abdominal aorta, the livers were isolated surgically and perfused in vitro using the recycling perfusion apparatus described previously (15, 16). The livers were generally isolated between 08:00 and 10:00 h and were perfused with a basal medium containing 3 g of bovine serum albumin (BSA)/dl, 100 mg glucose/dl, washed bovine erythrocytes (30% v/v) in Krebs-Henseleit bicarbonate buffer (pH. 7.4) (17). Oleate was infused as an albumin complex (6 g of purified BSA and 1197 µmol oleate/dl) at a constant rate (11.7 ml/h) to provide 140 µmol oleate/h in all experiments. A pulse of 10 mCi of ^3H_2O (with 60 µmol

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oleate) was added to the medium at zero time. The perfusions were maintained for 4 h. Under these conditions, the ambient concentration of FFA in the perfusate was maintained at 0.6 mM. Uptake of FFA was not different among groups (9.89 μmol/g liver per h). The flow rate of perfusate was maintained at 15–20 ml/min at 15 cm of hydrostatic pressure. Bile output and perfusion flow rates were monitored to assess viability during the 4 h of perfusion. No differences in bile output or perfusion flow rate were observed among groups.

Samples of perfusate were removed for analysis at the end of 2 and 4 h of perfusion. At the termination of the experiment, the livers were perfused with 60 ml of ice-cold 0.9% NaCl, cleansed of all extrahepatic tissue, blotted, and weighed. Samples of liver (1 g) were removed for analysis. Whole perfusates were centrifuged at low speed to sediment the red blood cells. A 60-ml sample of cell-free perfusate was obtained from the perfusate at the end of the experiment and used to isolate the nascent VLDL (VLDL secreted by the perfused liver) by ultracentrifugation, as described previously (18).

Analytical procedures

ApoB was determined in nascent VLDL and plasma using a radioimmunoassay similar to that used to determine apoA-I, as previously described (14, 19). Rat LDL (*d* 1.02–1.05 g/ml fraction) was used as the standard for the apoB assay. The LDL was 90% apoB as shown by densitometric scanning of Coomassie Blue-stained polyacrylamide electrophoretic gels (20). The diameters of VLDL particles isolated from liver perfusates were measured with a NICOMP model 370 submicron particle sizer (21).

To measure incorporation of ^3H_2O into perfusate and hepatic lipids, homogenized liver and other samples were extracted with chloroform–methanol 2:1 (v/v) (22). Aliquots of the washed chloroform extracts were separated into individual lipid classes by thin-layer chromatography on silica-gel G plates with a solvent mixture of light petroleum ether (b.p. 60–70°C)–diethyl ether–acetic acid 84:15:1 (by volume) (23). Aliquots of the chloroform extracts of the liver, whole perfusate, and plasma were saponified in 30% ethanolic KOH at 70°C for 2 h. FFA and cholesterol were extracted with petroleum ether after acidification, dried under nitrogen, and the lipid fractions were separated by TLC. The radioactivity in the lipid fractions was measured by liquid scintillation spectrometry with Ecolume as a scintillation cocktail. Synthesis of cholesterol was calculated from measured rates of ^3H_2O incorporation into saponified lipid using the perfusate ^3H_2O specific activity (24).

Chemical analyses of individual lipid classes were carried out by the methods of Rouser and Fleischer (25) for PL, Van Handel and Zilversmit (26) with modifications (27) for TG, and Rudel and Morris (28) for FC and CE. Protein was estimated by the method of Lowry et al. (29).

Enzyme assays

For determination of activities of cytosolic acetoacetyl-CoA (AcAc-CoA) thiolase (EC 2.3.1.9) and microsomal 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34), perfused and nonperfused livers from control and cholesterol fed animals were briefly flushed with ice-cold oxygenated 0.9% NaCl and were placed in the cold saline. Approximately 1-g portions of liver were removed as rapidly as possible and immediately homogenized in a buffer containing 50 mM potassium phosphate, 30 mM EDTA, 5 mM DTT, 200 mM NaCl, 50 mM NaF, pH 7.0, with a Dounce homogenizer. The homogenate was centrifuged at 10,000 *g* for 10 min, and the supernatant was recentrifuged for the same period. The supernatant of the second spin was centrifuged at 105,000 *g* for 60 min. The post-microsomal supernatant (cytosolic fraction) was used for determination of AcAc-CoA thiolase. The microsomal pellet was resuspended in 2 ml of the buffer (using a Teflon ball hand-held homogenizer). The cytosolic fraction and the microsomal suspension were stored at –70°C until assayed. AcAc-CoA thiolase activity was determined spectrophotometrically by measurement of AcAc-CoA cleavage after incubation in cuvettes at 37°C (20). HMG-CoA reductase activity was determined radiochemically by the method of Shapiro et al. (30), except that at the end of the incubation period [^{14}C]mevalonate was extracted three times with diethyl ether. The cytoplasmic and microsomal protein were determined by the method of Lowry et al. (29).

Statistical analysis

Values are reported as means ± SEM. Statistical evaluation of differences between treatment groups were made by using a one-way or a two-way ANOVA at the 95% probability level. Means were separated by a protected Fisher least significant difference test [PLSD] (31).

RESULTS

Food intake, animal and liver weights

No differences were observed in the daily intake (18–20 g/day) of food among all dietary groups during the experimental period. Mean body (300 g)

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and liver weights (14 g) and liver to body weight ratios were not significantly different for any of the groups. Overall, the liver to body weight ratios were 0.048 ± 0.006 (n = 54) without perfusion and 0.045 ± 0.001 (n = 45) after perfusion.

Hepatic and plasma lipid concentrations

No differences were observed in the concentration of phospholipid (PL) (28 μmol/g liver) in the livers of rats on any of the dietary groups. Hepatic TG, cholesterol, and CE lipid concentrations of rats on the various diets are shown in **Fig. 1**. Hepatic free cholesterol levels were moderately elevated at dietary concentrations of 0.5% cholesterol and above. Large increases in CE (100-fold) and TG (2- to 3-fold) content were observed with higher concentrations of cholesterol in the diet. Hepatic CE concentrations increased progressively with increase in dietary cholesterol.

Whole plasma lipid concentrations are shown in **Fig. 2**. Total plasma PL and cholesterol were unaffected by dietary cholesterol. Plasma TG levels were elevated by dietary cholesterol and were maximal with the 0.25% cholesterol supplement. As expected, plasma CE increased with cholesterol feeding but was maximal when 0.5% cholesterol was included in the diet.

Table 1 summarizes the effects of dietary cholesterol on the concentrations of the lipid components of plasma lipoproteins. Plasma concentrations of VLDL-TG, PL, FC, and CE were elevated over the 0.0% group when rats were fed 0.25% cholesterol or greater. Concentrations of LDL-PL, FC, and CE were also increased by cholesterol feeding. LDL was relatively enriched with cholesterol (12–54%), predominantly in the form of CE (6–138%). In contrast, the concentrations of the plasma HDL-PL and FC and to a lesser extent, CE, were reduced in rats fed higher levels of cholesterol.

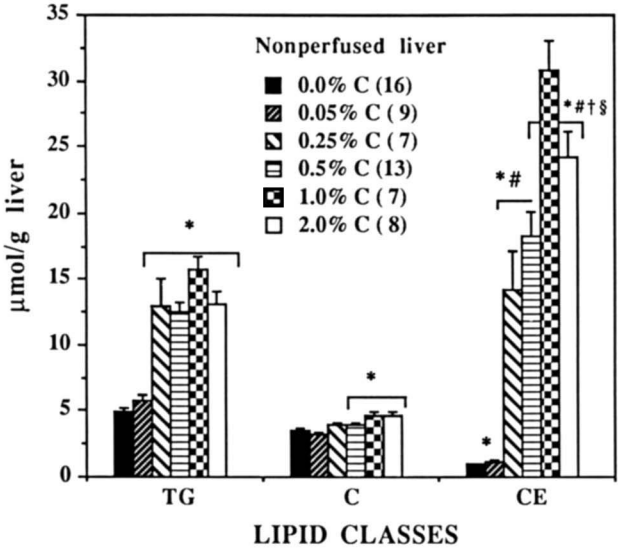

Fig. 1. Effects of dietary cholesterol on hepatic concentrations of triglyceride (TG), free cholesterol (C) and cholesteryl esters (CE). Male rats were fed a semipurified diet containing increasing concentrations of cholesterol for 21 days. Livers from these fed animals were isolated and assayed for lipid content as described in the Methods section. This figure represents the means ± SE for TG, C, and CE in nonperfused livers from cholesterol-fed rats. Number of animals per treatment group is indicated in parentheses. *Different from 0.0%; #different from 0.05%: †different from 0.25%; §different from 0.5% ($P < 0.05$).

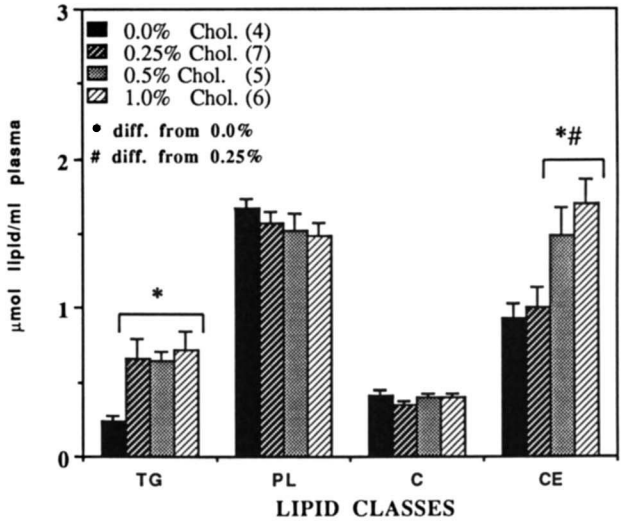

Fig. 2. Effects of dietary cholesterol on plasma lipid concentrations. Male rats were fed a semipurified diet containing increasing concentrations of cholesterol for 21 days (see Methods section). During surgery for isolation of livers, blood samples were obtained prior to cannulation of the portal vein, and plasma was separated. Analyses for plasma lipids were as described in the text. Data are means ± SE. Number of plasma samples per group is indicated in parentheses. *Different from 0.0%; #different from 0.25% ($P < 0.05$).

Output of nascent VLDL by the perfused liver

Hepatic VLDL lipid output, in the presence of a constant supply of oleate (0.6 mM), was increased in all lipid classes, as was secretion of protein associated with the VLDL (**Fig. 3**), by supplementing the diet with cholesterol; 0.05% and 0.1% dietary cholesterol increased output of PL and CE only. The output of all the lipid components and protein was similar for the livers from animals fed 0.5% cholesterol or higher. TG output by livers from cholesterol-fed rats was increased 2-fold by supplementation with 0.25% cholesterol and higher. Perfusate TG concentrations measured after 2 or 4 h of perfusion indicated that output of VLDL lipids was linear with time in all groups (data not shown).

Characteristics of nascent VLDL by the perfused liver

The effects of dietary cholesterol on the molar lipid ratios, output of VLDL protein and apoB, and particle

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TABLE 1. Effects of dietary cholesterol on concentrations of plasma VLDL, LDL, and HDL lipids

% Chol (n)	Triglyceride	Phospholipid	Cholesterol	Cholesteryl Esters
		μmol/ml plasma		
A. VLDL				
0.00 (14)	0.23 ± 0.02	0.08 ± 0.01	0.03 ± 0.01	0.04 ± 0.00
0.05 (7)	0.30 ± 0.06	0.11 ± 0.01	0.04 ± 0.00	0.05 ± 0.01
0.25 (7)	0.45 ± 0.06[a]	0.22 ± 0.03[a,b]	0.09 ± 0.01[a,b]	0.44 ± 0.08[a,b]
0.50 (13)	0.38 ± 0.03[a]	0.21 ± 0.01[a,b]	0.10 ± 0.01[a,b]	0.47 ± 0.05[a,b]
1.00 (6)	0.44 ± 0.05[a]	0.29 ± 0.01[a,b]	0.16 ± 0.01[a,b]	0.82 ± 0.07[a,b]
2.00 (8)	0.27 ± 0.03	0.20 ± 0.00[a,b]	0.08 ± 0.01[a,b]	0.46 ± 0.04[a,b]
B. LDL				
0.00 (12)	0.12 ± 0.01	0.13 ± 0.02	0.07 ± 0.01	0.23 ± 0.03
0.05 (6)	0.13 ± 0.02	0.12 ± 0.02	0.08 ± 0.01	0.24 ± 0.03
0.25 (7)	0.12 ± 0.01	0.11 ± 0.03	0.07 ± 0.01	0.32 ± 0.04[a]
0.50 (11)	0.13 ± 0.01	0.17 ± 0.01[a]	0.10 ± 0.01[a]	0.51 ± 0.04[a,b]
1.00 (7)	0.15 ± 0.01	0.18 ± 0.01[a]	0.10 ± 0.01[a]	0.54 ± 0.05[a,b]
2.00 (6)	0.10 ± 0.02	0.16 ± 0.01[a]	0.11 ± 0.01[a]	0.48 ± 0.06[a,b]
C. HDL				
0.00 (12)	0.01 ± 0.02	0.54 ± 0.05	0.13 ± 0.01	0.77 ± 0.07
0.05 (6)	0.01 ± 0.00	0.46 ± 0.09	0.10 ± 0.01	0.65 ± 0.11
0.25 (6)	0.03 ± 0.01	0.37 ± 0.02[a]	0.07 ± 0.01[a]	0.69 ± 0.03
0.50 (11)	0.01 ± 0.00	0.39 ± 0.05[a]	0.07 ± 0.01[a]	0.60 ± 0.05
1.00 (7)	0.02 ± 0.00	0.28 ± 0.02[a,b]	0.06 ± 0.01[a]	0.58 ± 0.03
2.00 (6)	0.01± 0.00	0.29 ± 0.07[a,b]	0.05 ± 0.01[a,b]	0.42 ± 0.07[a]

VLDL, LDL, and HDL were isolated by ultracentrifugation from plasma, and lipid analyses were determined as described in Methods. The data represent means ± SE. The number of samples per treatment is given in parentheses.
[a]Different from 0.0% cholesterol group; [b]different from 0.05% ($P < 0.05$).

size of VLDL secreted by the perfused liver are shown in **Table 2**. VLDL lipid molar ratios were calculated, and VLDL particle sizes were measured to estimate whether the greater secretion rate of VLDL by the liver was due to increase in particle number, or in diameter, with cholesterol feeding. The molar ratios of the surface lipids (PL/FC) of the nascent VLDL were not different. However, the molar ratio of the core lipids (TG/CE) decreased dramatically. Clearly, although output of both VLDL-TG and CE increased significantly with increasing dietary cholesterol (Fig. 3), the relatively greater increase in CE content reduced the molar ratio TG/CE. It is of particular interest that the molar ratios of the total surface to core lipid components (PL + FC/TG + CE) remained constant regardless of the concentrations of dietary cholesterol, suggesting similarities in VLDL particle size. Consistent with increased VLDL total protein secretion (Table 2), dietary cholesterol stimulated VLDL apoB secretion by the perfused liver.

The diameters of nascent VLDL are also shown in Table 2. Cholesterol feeding under these experimental conditions did not affect nascent VLDL particle size, consistent with the similarity of the surface/core lipid ratios. The increase in lipid and apoB output, the constancy in surface to core molar ratios, and the similarity of diameters of the nascent VLDL particles indicate that cholesterol feeding increased VLDL particle number without altering particle size. Clearly, the secreted VLDL particles had similar volumes with cholesterol feeding, although the composition of the particles was different, because of the increase in CE content relative to that of TG.

Characteristics of plasma VLDL of cholesterol-fed rats

Plasma VLDL lipid molar ratios and concentration of VLDL protein of cholesterol-fed rats are summarized in **Table 3**. The molar ratios of the plasma VLDL surface lipids (PL/FC) were not different in the various dietary groups. The molar ratios of the plasma VLDL surface to core lipids (PL + FC/TG + CE) were relatively unchanged. The characteristics of plasma VLDL and the nascent VLDL secreted by the perfused livers were quite similar, suggesting that the livers from these animals secreted VLDL that reflected both in vivo hepatic composition (primarily TG and CE) as well as that newly synthesized, even when perfused in vitro with exogenous oleic acid.

Plasma VLDL total protein was increased (12–61%) by dietary cholesterol (Table 3). No further increase was observed above 0.25% cholesterol in the diet. As observed with nascent VLDL, the ratio of total plasma VLDL protein to plasma VLDL-TG was not affected by dietary cholesterol. Furthermore, concentrations of total plasma apoB were not affected by dietary cholesterol under our experimental conditions.

Hepatic lipid concentrations after perfusion

The concentrations of hepatic lipids were determined following perfusion and were compared to those levels attained in vivo (i.e., prior to perfusion).

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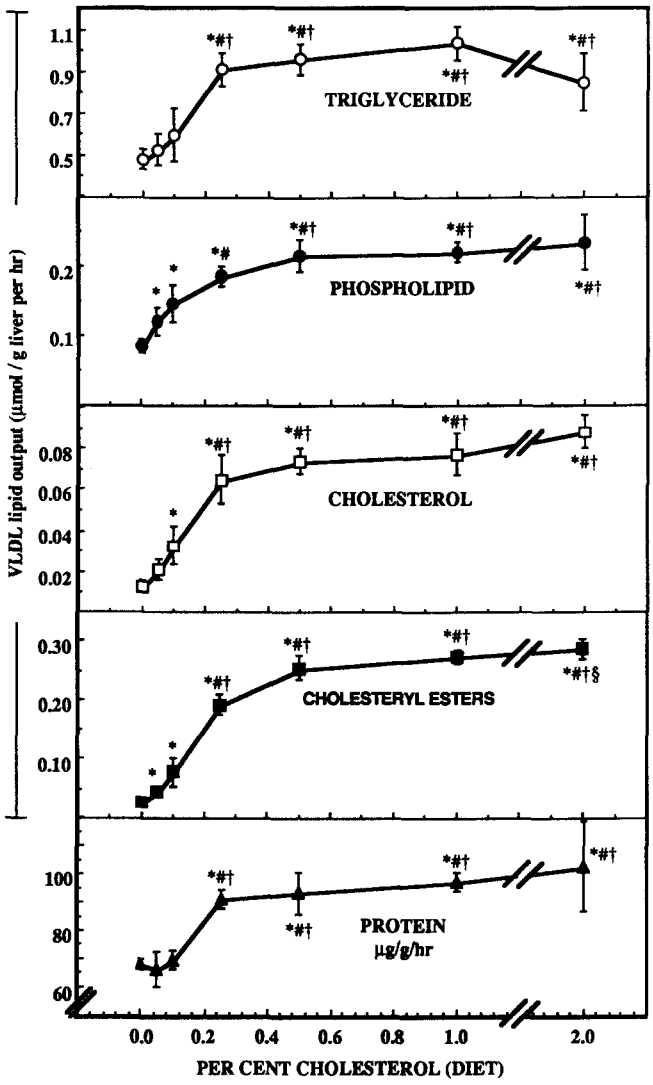

Fig. 3. Effects of dietary cholesterol on secretion of VLDL lipids and protein by the isolated perfused liver. Perfusate samples for analysis of VLDL lipids and apolipoproteins were obtained at the end of 4 h of perfusion. VLDL was isolated by ultracentrifugation (d < 1.006 g/ml). VLDL lipid and protein analyses were measured as indicated in the Methods section. Data represent means ± SE. Number of VLDL samples per group is indicated elsewhere in Table 2. *Different from 0.0%; #different from 0.05%: †different from 0.25%; §different from 0.5% ($P < 0.05$).

Significant changes as a result of perfusion are shown in **Fig. 4**. Hepatic PL and FC concentrations were not different after perfusion (data not shown). TG concentrations in livers of rats on the 0.0% and 0.05% cholesterol in the diet were increased by perfusion with oleate. The hepatic TG levels did not increase further, when livers from rats fed the higher amounts of cholesterol were perfused with oleic acid, perhaps related to the higher secretory rates of TG (Fig. 2). These data suggest, therefore, that livers from the 0.0% and 0.05% cholesterol groups accumulated TG, while secreting less TG during perfusions with oleate; in contrast, livers from rats on the diets supplemented with higher amounts of cholesterol in the diet secreted more of the infused fatty acid as newly synthesized TG, since TG did not accumulate. The concentration of hepatic CE in livers of cholesterol-fed rats (0.25% or greater) was reduced during perfusion, suggesting that the secreted CE may have been derived to a significant extent from the hepatic CE pool for VLDL formation and secretion.

Linear regression analysis of data from all perfusions indicated that a positive correlation can be constructed ($r = 0.696$, $P < 0.0001$) between hepatic CE concentration and VLDL-TG secretory rates (**Fig. 5**). Similar conclusions were obtained in previous experiments, in which hepatic CE content was decreased with lovastatin (5, 6). However, in the current experiments the relationship may be more appropriately defined by a hyperbolic function. Group means for output of VLDL-TG were proportional to the mean concentrations of hepatic CE up to about 6 µmol/g liver (Fig 5. insert). Further evaluation in hepatic CE content did not elicit additional output of TG under our perfusion conditions. It may be that insufficient substrate (oleate) was available for TG synthesis in these experiments and that had more FFA been provided, output of VLDL-TG would have continued proportionally to hepatic CE concentration.

Effect of dietary cholesterol on hepatic lipid synthesis

Synthesis of cholesterol was followed by incorporation of ^3H_2O into total cholesterol, and the activity of HMG-CoA reductase in livers from intact animals (panel A, **Fig. 6**) and in perfused livers (panel B, Fig. 6) from control and cholesterol-fed rats. The activity of cytosolic acetoacetyl-CoA thiolase was also measured in the nonperfused and perfused livers (panel C, Fig. 6). Synthesis of cholesterol (^3H_2O incorporation), the activity of microsomal HMG-CoA reductase, and the activity of cytosolic AcAc-CoA thiolase were all depressed by cholesterol feeding, as expected (32). The HMG-CoA reductase activity of perfused livers was moderately elevated over that of nonperfused livers (33), despite the substantial pool of hepatic cholesterol. Activity of HMG-CoA reductase was reduced even with 0.05% cholesterol in the diet, while the activity of AcAc-CoA thiolase was not affected. AcAc-CoA thiolase activity did not change during perfusions.

DISCUSSION

The synthesis of TG and its secretion in the VLDL by livers from normal fed rats is a function of the concentration of free fatty acid (FFA) perfusing the liver (1, 2). Our laboratory had proposed earlier that lipid

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TABLE 2. Molar lipid ratios, output of VLDL protein, VLDL apoB, and particle sizes of VLDL secreted by perfused livers from cholesterol-fed rats

VLDL	% Cholesterol in Diet 0.0 (14)	0.05 (8)	0.1 (4)	0.25 (7)	0.5 (13)	1.0 (7)	2.0 (8)
Phospholipid:cholesterol (PL/FC)	2.5 ± 0.3	3.0 ± 0.6	2.7 ± 0.1	2.6 ± 0.5	2.3 ± 0.7	2.5 ± 0.2	2.2 ± 0.3
Triglyceride:cholesteryl esters (TG/CE)	18.8 ± 1.2	10.9 ± 1.4[a]	8.5 ± 2.4	4.7 ± 0.4[a,b]	3.8 ± 0.3[a,b]	3.8 ± 0.3[a,b]	3.1 ± 0.3[a,b,c]
Particle size (diameter in nm)	90.8 ± 4 (5)	91.7 ± 6 (6)	ND	ND	87.4 ± 4 (5)	ND	82.9 ± 23 (5)
Surface:core ratio $\frac{PL+FC}{TG+CE}$	23.9 ± 0.9	26.8 ± 0.7	29.9 ± 0	24.8 ± 0.8	26.0 ± 1.1	24.1 ± 0.9	29.5 ± 0.8
VLDL protein output (μg/g)	52 ± 3	49 ± 9	54 ± 5	87 ± 4[a,b]	90 ± 11[a,b]	96 ± 5[a,b]	104 ± 24[a,b]
ApoB (μg/g/h)	18.7 ± 3 (5)	ND	40.6 ± 4.5[a] (4)	47.4 ± 2[a] (6)	52.0 ± 5[a] (4)	49.0 ± 3[a] (5)	ND

Rats were fed a semipurified diet containing increasing concentrations of cholesterol for a period of 3 weeks. On the morning of the experiments, livers from these animals were surgically isolated and perfused with oleic acid and medium as described in Methods. VLDL, apoB, and protein were isolated and analyzed as described in the text. The data are given as means ± SE representing the calculated ratios for VLDL-lipids, measured VLDL particle sizes (as vesicles), and immunoprecipitable apoB. Number of measurements per treatment group is given in parentheses.

[a]Different from 0.0%; [b]different from 0.05%; [c]different from 0.25% cholesterol-fed groups ($P < 0.05$).

surface components (PL or FC) can become rate-limiting for transport of TG in the VLDL (4), and we have also recently provided evidence that cholesterol is required, and may, under certain conditions, be rate-limiting for formation and secretion of VLDL-TG (5, 6). It was furthermore postulated that cholesterol, when provided to the normal liver, can be a stimulant for VLDL formation. That this indeed occurs is suggested by the present study in which secretion of TG, as well as other lipid components of the VLDL, was increased, in the presence of fatty acid, by livers from cholesterol-fed rats. The output of VLDL protein also increased parallel to that of VLDL-TG. The ratio of PL to FC of the surface was constant as was the ratio of surface to core lipid components of the secreted VLDL, with increase in VLDL output, while the composition of the core lipids changed such that the ratio of CE to TG increased approximately 6-fold with cholesterol feeding. The composition of the secreted particle, however, differed from that when VLDL formation was stimulated by FFA only (34). When oleate is the primary stimulus for VLDL formation, the particle size increases as the supply of oleate increases, i.e., core to surface ratio increases, but the surface lipid ratio (PL/FC) is nevertheless constant. This may occur because of a limitation of cholesterol as a VLDL structural component, which is plentiful in the liver of a cholesterol-fed rat and potentially available for VLDL fabrication.

We reported previously that treatment of rats with lovastatin, an inhibitor of cholesterol synthesis and HMG-CoA reductase, decreased the content of hepatic CE and secretion of all lipid components of the VLDL (5, 6). Furthermore, addition of cholesterol to the diet containing lovastatin, or addition of human LDL to the medium perfusing the isolated liver in vitro, reversed these effects, i.e., it increased hepatic CE concentration and stimulated the secretion of the VLDL lipids (5). We postulated that a metabolic pool of cholesterol in the liver provided cholesterol for formation of VLDL and for transport of TG. In the present study we observed that addition of cholesterol to diets fed to

TABLE 3. Plasma VLDL lipid molar ratios, concentrations of VLDL protein, and total plasma apoB in cholesterol-fed rats

Analysis	% Cholesterol in Diet 0.0 (14)	0.05 (7)	0.25 (7)	0.5 (13)	1.0 (7)	2.0 (7)
Phospholipid:cholesterol (PL/FC)	2.54 ± 0.19	2.72 ± 0.15	2.39 ± 0.13	2.12 ± 0.13	2.10 ± 0.17	2.37 ± 0.11
Triglyceride:cholesteryl esters (TG/CE)	6.49 ± 0.86	5.37 ± 0.50	1.11 ± 0.10[a,b]	0.88 ± 0.10[a,b]	0.60 ± 0.11[a,b]	0.60 ± 0.07[a,b]
Surface:core ratio $\frac{PL+FC}{TG+CE}$	45.3 ± 3.3	47.9 ± 6.5	35.1 ± 2.7	37.6 ± 1.6	36.4 ± 1.4	37.9 ± 2.7
VLDL protein output (μg/ml)	5.0 ± 0.4 (7)	5.6 ± 0.9 (5)	7.2 ± 0.8 (4)[a,b]	8.1 ± 0.4 (6)[a,b]	8.1 ± 0.9 (4)[a,b]	7.4 ± 0.9 (5)[a,b]
Plasma apoB (mg/dl)	15.5 ± 1.6 (8)	ND	17.2 ± 1.3 (10)	20.2 ± 1.2 (7)	18.7 ± 1.8 (10)	ND

Plasma for analysis of VLDL and apolipoproteins was obtained from most rats prior to removal of livers for perfusion. VLDL was isolated from plasma by ultracentrifugation (d < 1.006 g/ml). VLDL lipid analysis, protein, and apolipoproteins were measured as indicated in Methods. The ratios represent calculated values for plasma VLDL lipid values shown in Table 1. The number of measurements per group is given in parentheses. ND, not determined.

[a]Different from 0.0%; [b]different from 0.05% ($P < 0.05$).

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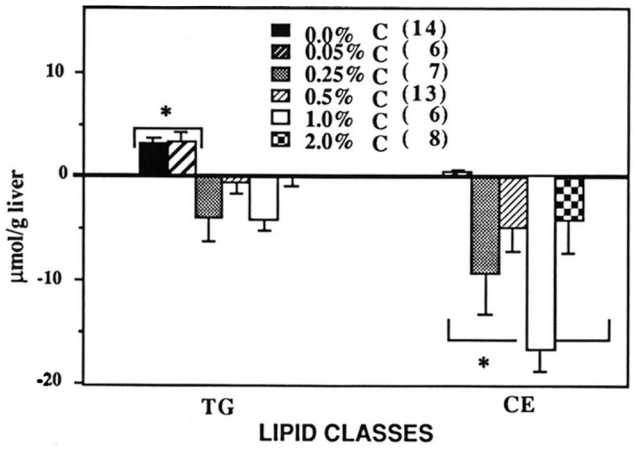

Fig. 4. Mean differences between hepatic concentrations of TG and CE of nonperfused and perfused livers from cholesterol fed rats. Livers were isolated from control or cholesterol-fed rats and were either assayed directly for lipid concentrations or were first perfused for 4 h with oleic acid, and then assayed for lipid concentrations. The data presented are the calculated differences ± SE of lipid concentrations between the nonperfused and perfused rat livers. Number of livers per treatment group is indicated in the legend to Fig. 1. *Significant increase or decrease from 0.0% and 0.05% cholesterol ($P < 0.05$).

normal rats increased hepatic FC and CE content and secretion of VLDL lipid and associated protein, including immunoreactive apoB, by perfused livers isolated from these rats.

Two interpretations of these data are possible. Free cholesterol derived from a metabolic pool (perhaps located in the endoplasmic reticulum) is required for formation of the surface of VLDL particles to transport of TG; depletion or repletion of this putative pool alters the rate at which VLDL can be formed and secreted. In the lovastatin study (5) treatment with the drug or supplementation with dietary cholesterol, or LDL in the medium did not affect the concentration of total hepatic FC. In the present study, with rats fed increasing quantities of cholesterol, changes in FC content of the liver were apparent when rats were fed 0.5% cholesterol or greater. Most of the hepatic FC is located in plasma membranes (35); the increase in concentration of FC, therefore, may represent a metabolic pool of cholesterol that is also substrate for esterification. The hepatic CE content may be a reasonable index of the contraction or expansion of a putative metabolic pool of FC, particularly at lower levels of cholesterol feeding, in which the hepatic CE was elevated, but differences in FC could not be detected. While this is a plausible hypothesis, direct measurements of subcellular cholesterol content changes are not available. In our studies with lovastatin, secretion of VLDL-TG by the liver, supplied with a constant amount of oleate, was observed by us to be proportional to the content of hepatic CE. The present study, with livers from rats fed varying amounts of cholesterol up to 0.5%, also suggests a positive correlation between hepatic CE concentration and VLDL-TG secretion. In these experiments when higher levels of cholesterol were fed, and increasingly greater amounts of CE accumulated in the liver, the supply of FFA may have been rate limiting for TG synthesis and TG transport, so that a linear response at the higher hepatic concentrations of CE was not obtained.

A second interpretation is that CE, even though its concentration may reflect the size of a metabolic pool of FC, may itself be required for VLDL formation and, therefore, may itself be rate determining. Hepatic acyl-CoA:acyltransferase (ACAT) activity is localized in the rough endoplasmic reticulum (36). The primary neutral core lipid, TG, is added in the cisternae of the smooth endoplasmic reticulum (37) to the nascent apoB which may be stabilized by newly synthesized CE during transit. Consistent with this is the finding that depletion of cellular CE by prolonged treatment with an ACAT inhibitor diminishes the rate of TG secretion by CaCo2 cells (38, 39) and apoB secretion by HepG2 cells (40). Moreover, 24-h exposure of HepG2 cells to lovastatin, which also partially depletes the CE content of the cells, reduces secretion of apoB (40).

Davis and Boogaerts (7) reported that cultured hepatocytes from female rats fed a diet containing 2% cholesterol and 20% olive oil for 8 weeks secreted less TG and more CE into medium lacking substrate amounts of exogenously supplied FFA. The resultant VLDL particles were enriched with CE and contained less TG than particles secreted by hepatocytes derived from rats whose diet was not supplemented with cholesterol. Their conclusion, that the enrichment of the core of each particle by CE was at the expense of TG, is in agreement with our conclusions. However, when exogenous fatty acid was available to the liver and TG synthesis was enhanced, secretion of TG was, in fact, stimulated (as was CE) over that of livers from rats fed a diet not supplemented with cholesterol. Since we observed that VLDL particle size did not vary with the cholesterol content of the diet, lipid synthesis, or hepatic lipid content, we can conclude that when increased amounts of CE are transported in VLDL it is at the expense of TG in the core. Even so, with markedly increased CE content, TG nevertheless remains the major core lipid component of the VLDL. Additional particles are required, and are assembled, to accommodate the presumed increase in TG synthesis and its transport in VLDL. For this purpose additional apoB is required. This additional requirement is either fulfilled by increased synthesis of apoB or by decreased intracellular degradation, a process proposed by Dixon, Furukawa, and Ginsberg (41) to account for regulation of the pool of this apolipoprotein whose gene is constitutively expressed (42).

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We had reported previously that the stimulation of hepatic TG synthesis with concomitant formation and secretion of VLDL lipid in response to exogenous fatty acid (oleic acid) was attended by an increase in associated apolipoproteins secreted in the VLDL particles; the secretion of apoB (-100 and -48) was increased by about 40% and 150% in the VLDL derived from livers of fed and fasted rats, respectively. Secretion of newly synthesized apoB, however, was stimulated by fatty acids only in livers from fasted rats (20). On a high carbohydrate diet, as in this study, enhanced hepatic synthesis of VLDL-TG has been observed (43–45). Under these conditions, increased synthesis of VLDL-TG is not usually accompanied by enhanced production rates for VLDL-apoB (46). Eisenberg and Levy (47) reported that the apoB content per lipoprotein particle was constant regardless of the differences in molecular weights of the particles, and that the concentration of total apoB was related to particle number. It is clear in our present studies that hepatic apoB secretion is stimulated by cholesterol feeding, which may also reflect increased synthesis of that apolipoprotein.

Hepatic concentration of TG was increased by cholesterol feeding, and this was associated with hypertriglyceridemia. The hypertriglyceridemia may, in part, have resulted from stimulation of output of VLDL-TG, as shown in the present perfusion studies. The occurrence of both events suggests that TG synthesis is increased. Whether this is the result of increased substrate (fatty acid) availability for TG synthesis because of stimulated de novo synthesis of fatty acids or decreased fatty acid oxidation remains to be determined, since no differences in the uptake of oleate by the perfused liver were apparent. It is also possible that the rate of TG synthesis per se is stimulated by dietary cholesterol. It is of interest in this regard that inhibition of carnitine palmitoyltransferase (EC 2.3.1.21, CPT), a rate-limiting enzyme in fatty acid oxidation, induced hepatic HMG-CoA reductase (32). It had been established earlier that either provision of FFA to the liver (1, 2) or inhibition of fatty acid oxidation (33) increased VLDL-TG secretion, HMG-CoA reductase activity, and cholesterol synthesis. It therefore seems logical to postulate that provision of excess cholesterol to the liver, which stimulates VLDL-TG secretion, may, in part, do so by inhibition of fatty acid oxidation, making more fatty acid available for esterification to TG. In a previous study from this laboratory (5, 6), when cholesterol was included in the diet of lovastatin-fed rats, the mass of TG in the liver was increased and the output of VLDL was returned toward control levels; the suggestion from those data was that cholesterol may stimulate synthesis of TG in addition to providing sterol for formation of VLDL.

During perfusion with oleate, TG accumulated in livers from rats fed the cholesterol-free or 0.05% cholesterol-supplemented diet, whereas livers from rats fed the higher levels of cholesterol, and already possessing elevated levels of hepatic TG, did not accumu-

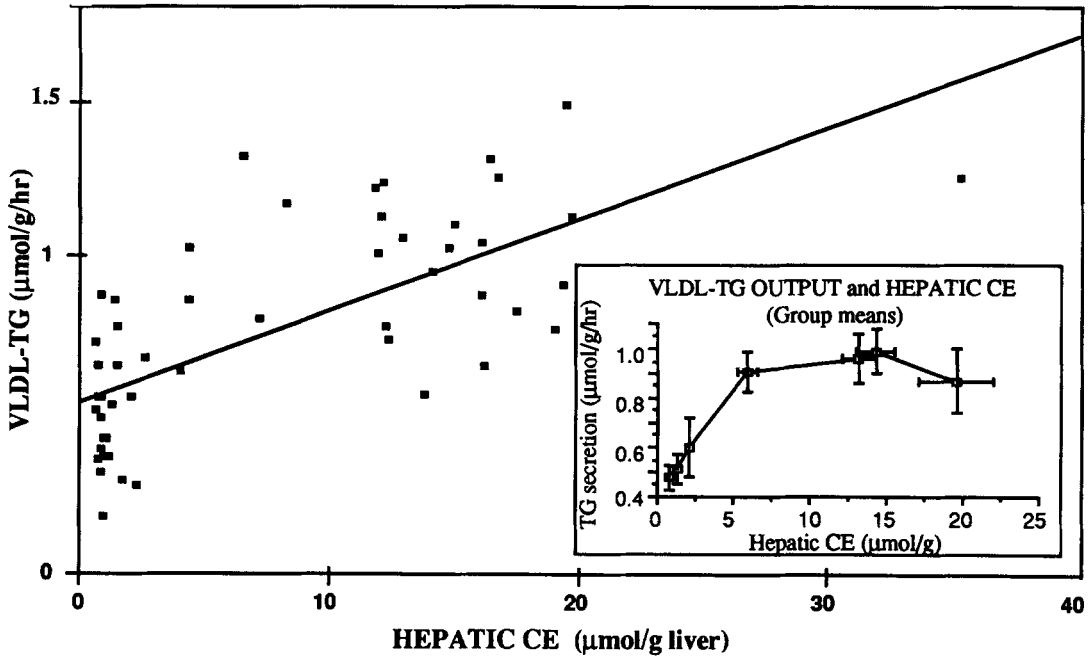

Fig. 5. Relationship between VLDL-TG secretion and hepatic CE concentration. Secretion of VLDL-TG was plotted as a function of hepatic CE concentrations for the corresponding liver. The insert depicts a plot of the group means ± SE for VLDL-TG output and hepatic CE concentrations.

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late any additional TG during perfusion. In these latter groups, secretion of VLDL-TG and other lipids was greater than that of controls. Under these perfusion conditions, newly synthesized TG may not have accumulated additionally because cholesterol was readily available (derived from the CE pool) for fabrication and secretion of VLDL, and which might have been limited in those livers of rats fed the cholesterol-free or 0.05% diet. Additionally, it is possible that, in order to secrete the excess CE in the VLDL, TG is still required as the major component of the VLDL core for the outward transport of CE from the liver. The marked decline in hepatic CE can be partly accounted for by the increased level of secretion of CE in the VLDL particles; the remainder was probably secreted in bile as cholesterol or as bile acids.

Hepatic cholesterol synthesis and microsomal HMG-CoA reductase activity were suppressed at all levels of cholesterol feeding, as would be expected (2). The activity of microsomal HMG-CoA reductase was moderately elevated in livers perfused for 4 h in the presence of oleic acid. This suggested that oleic acid stimulated the activity of microsomal HMG-CoA reductase, as has been reported previously (17, 48, 49), but not to a degree sufficient to overcome the down-regulation of dietary cholesterol on the enzyme under these conditions. It is also remarkable that as little as 0.05% cholesterol in the diet inhibited the activity of hepatic microsomal HMG-CoA reductase activity, but did not affect cytosolic acetoacetyl-CoA thiolase activity.

The suggested requirement for and potential regulatory influence of cholesterol on hepatic secretion of the VLDL, as proposed (4–6), are supported by our present studies. This is apparent in the similarities of the VLDL particle diameters, supported by the constancy of the ratio of the VLDL surface lipids (PL/FC) despite a wide range of VLDL core composition. Even when the particle size is altered, as when the liver produces VLDL particles from FFA of differing chain length and degree of saturation, this ratio is not altered (24). We have also reported ratios similar to those in this study (with erythrocytes) when livers were perfused without erythrocytes (24). Therefore, if the

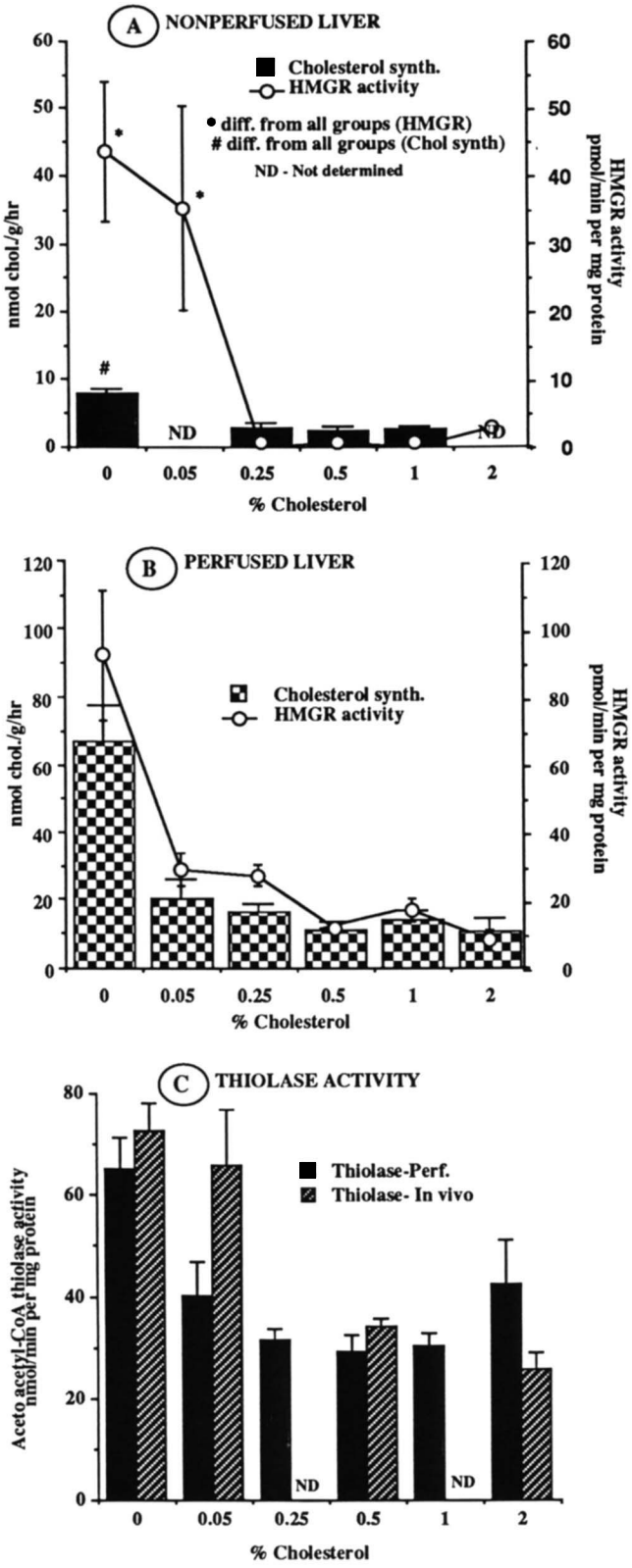

Fig. 6. Effects of dietary cholesterol feeding on cholesterol synthesis, and activities of microsomal HMG-CoA reductase, and cytosolic acetoacetyl-CoA thiolase. Rats were fed varying concentrations of cholesterol, as described in the text, for 21 days. The intact animal was injected with ^3H_2O (i.p.) to measure hepatic cholesterol synthesis after 2 h in vivo. Livers from fed animals were also isolated and perfused in vitro for 4 h (see Methods) with ^3H_2O in the medium to measure lipid synthesis. Synthesis of cholesterol is presented as nmol ^3H incorporated per g liver per hour ± SE into cholesterol. The same samples from perfused and nonperfused (in vivo) livers provided the microsomal and cytosolic fractions used to assay for HMG-CoA reductase and AcAc-CoA thiolase activities. The minimal number of samples is reported in the legend to Fig. 1 for the in vivo experiments and in Table 2 for the perfused liver experiments. Panel A presents cholesterol synthesis and reductase activity in vivo, while panel B shows related data for the perfused liver. Panel C indicates thiolase data in vivo and in the perfused liver.

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VLDL acquires cholesterol from erythrocyte membranes, as discussed by Faergeman and Havel (50), then PL must also be acquired in appropriate ratio. This seems unlikely. The contention by Hamilton, Moorehouse, and Havel (51), based on observations that the cholesterol content of VLDL may be variable and is therefore, unlikely to be coupled with regulation of VLDL formation, is not supported by our findings; this apparent controversy remains to be resolved.

The average particle size of 82–92 nm for the nascent VLDL secreted by the livers, as determined with the particle sizer, is larger than values reported for VLDL in plasma or associated with hepatic Golgi (51) (the upper limit being about 75 nm). The larger size we observed may reflect the hepatic formation of VLDL when stimulated by the presence of fatty acid in the medium. Most other measurements of VLDL particles secreted in vitro were made from liver perfusates to which an exogenous source of FFA had not been added. We did not measure the particle sizes of plasma VLDL in the current experiments. However, based on the greater ratio of surface lipids to core lipids, the particle size is probably smaller than the perfusate counterpart, perhaps the result of partial metabolism during circulation in vivo.

A number of studies have reported effects of cholesterol feeding on VLDL composition. Although differences in feeding schedules, animal species, and differences in diet composition (fat and cholesterol, particularly) exist, a complicating factor in some studies with increased dietary cholesterol has been the addition of propylthiouracil (PTU) in the diet, with the resulting hypothyroid state. In the studies of DeLamatre and Roheim (52), in which rats were fed cholesterol containing diets with and without PTU, plasma concentrations of lipoproteins with densities in the VLDL, IDL, and LDL ranges were increased with cholesterol feeding alone. These observations are not unlike the pattern we presently describe. When PTU was also included in the diet, IDL and LDL levels were further increased, although VLDL levels were not different from those of chow-fed controls. Our laboratory has previously reported the increased secretion of VLDL by perfused livers from hypothyroid rats (53). It is quite clear that the thyroid status of the animal significantly influences the assembly and composition of lipoprotein particles transporting lipid from the liver (3). Swift et al. (54) examined the composition of VLDL particles in the hepatic Golgi of rats fed cholesterol and PTU and concluded that the liver was responsible for the increase of IDL and LDL particles in the plasma. Our data are consistent with the primary role of the liver in the production of lipoprotein particles with a composition similar to that found in the plasma of the cholesterol fed animals, as are also the data of Noel et al. (55) for lipoproteins produced by perfusion livers from rats fed cholesterol and PTU. To transport CE and TG, perfused livers from guinea pigs were found to form IDL-like particles in excess of VLDL (56). It is probable that formation of VLDL-type particles was not observed in that study because FFA were not provided for the stimulation of TG synthesis and formation of VLDL, as in our study.

In our current study, with diets containing 5% corn oil, it was demonstrated that hypertriglyceridemia resulted from cholesterol feeding, and that this was a consequence of the transport of excess CE from the liver in VLDL, for which process an increased supply of TG was necessary. The degree to which cholesterol may induce hypertriglyceridemia may also be related to the chemical make-up and quantity of fat in the diet. It was demonstrated recently by Van Heek and Zilversmit (57) that feeding cholesterol with hydrogenated coconut oil, but not olive oil, induced a marked hypertriglyceridemia, resulting in part from increased production of VLDL by the liver. Our observations also indicate that the degree of hypertriglyceridemia is a function of cholesterol content of the diet, and suggest that the cholesterol-induced hypertriglyceridemia may arise to a significant extent from increased secretion of VLDL-TG by the liver. Clearly, synthesis and transport of TG and CE in the VLDL are inextricably linked, and knowledge of the regulation of this hepatic transport process is fundamental to an understanding of the mechanisms of production of hypertriglyceridemia and hypercholesterolemia.

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