Chylomicron metabolism during dietary-induced hypercholesterolemia in dogs

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Abstract The metabolism of [1-^{3}H]retinol- and [4-^{14}C]cholesterol-labeled chylomicrons was studied in normal and cholesterol-fed dogs in order to estimate the relative contribution of chylomicron remnant cholesterol to diet-induced hypercholesterolemia. The plasma $t_{1/2}$ of intravenously administered $S_f > 400$ chylomicrons, S_f 20–400 chylomicrons, and whole lymph doubly labeled with [1-^{3}H]retinol and [4-^{14}C]cholesterol was not significantly prolonged in hypercholesterolemic recipients. When $S_f > 400$ chylomicrons were administered intravenously, 90% of the radioactivity was cleared from the plasma of both normal and cholesterol-fed dogs within 1 hr and 68 ± 18% appeared in the liver within approximately 2 hr in normal dogs and 4 hr in hypercholesterolemic dogs. The use of the retinol-labeling technique for intestinal lipoproteins provided evidence that some LDL, but essentially none of the HDL_c, was derived from $d < 1.006$ g/ml lymph lipoproteins. The failure of significant radioactivity to accumulate in the plasma compartment of hypercholesterolemic dogs after intravenous administration of doubly labeled chylomicrons and the relatively efficient uptake of radioactivity by the liver indicate that the dietary-induced hypercholesterolemia in dogs is not the result of impaired hepatic removal of chylomicron remnants.—**Melchior, G. W., R. W. Mahley, and D. K. Buckhold.** Chylomicron metabolism during dietary-induced hypercholesterolemia in dogs. *J. Lipid Res.* 1981. **22:** 598–609.

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Dietary-induced hypercholesterolemia is required for the accelerated production of atherosclerosis in most animal models of the disease. The changes that occur in the plasma cholesterol concentration and lipoprotein distribution have been well characterized for several species (1–5) and, although there is marked individual variation, certain basic changes are common to most species studied. These include an increase in the concentration and cholesterol content of low density lipoproteins (LDL), a decrease in the concentration of high density lipoproteins (HDL), and the appearance of beta-migrating very low density lipoproteins (β-VLDL). In addition, the dog, swine, and rat develop HDL_c (1), α-migrating lipoproteins which accumulate in the $d < 1.087$ g/ml plasma fraction.

It is noteworthy that β-VLDL does not become a prominent cholesterol-carrying fraction in foxhounds until plasma cholesterol levels of 750 mg/dl are attained, and in the absence of β-VLDL, atherosclerosis fails to develop. Furthermore, the β-VLDL of cholesterol-fed dogs and other animals are the only naturally occurring plasma lipoproteins that will cause massive cholesteryl ester accumulation in macrophages (6, 7), a potentially important cell type in experimental atherosclerosis. It is therefore critical to determine the origin and function of β-VLDL in order to understand completely the dietary-induced disease.

Ross and Zilversmit (8) have demonstrated that, concomitant with the onset of hypercholesterolemia in the rabbit, there is an impaired hepatic clearance of cholesteryl ester-rich chylomicron remnants from the plasma. They estimated that these remnants could account for as much as two-thirds of the $d < 1.006$ g/ml cholesterol pool and concluded that the cholesteryl esters associated with lipoproteins of $d < 1.006$ g/ml in hypercholesterolemic rabbits were primarily of intestinal origin. However, recent studies

Abbreviations: VLDL, (very low density lipoproteins) those lipoproteins of $d < 1.006$ g/ml which migrate with the $alpha_2$ globulins during agarose electrophoresis; β-VLDL, lipoproteins of $d < 1.006$ g/ml which migrate with the beta globulins during agarose electrophoresis; IDL, (intermediate density lipoproteins) beta-migrating lipoproteins of d 1.006–1.020 g/ml; LDL (low density lipoproteins) beta-migrating lipoproteins of d 1.020–1.087 g/ml; HDL_t, alpha-migrating lipoproteins of d 1.006–1.087 g/ml; HDL_c, alpha-migrating lipoproteins of d 1.006–1.087 g/ml isolated from hypercholesterolemic dogs; $S_f > 400$ and S_f 20–400 are subfractions of the $d < 1.006$ g/ml lipoproteins isolated by preparative ultracentrifugation as described in Methods: $S_f < 20$ refers to lipoproteins of d 1.006–1.21 g/ml. The term chylomicrons as used herein refers solely to lipoproteins presumed to be of intestinal origin. TLC, thin-layer chromatography.

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with hypercholesterolemic rats (9–11) demonstrated that the livers from those animals were capable of synthesizing and secreting cholesteryl ester-rich lipoproteins of d < 1.006 g/ml. These studies suggest a hepatic origin for β-VLDL in that species. We report here the results of studies designed to determine whether the β-VLDL cholesterol pool that accumulates in cholesterol-fed foxhounds is derived from chylomicron remnants, and whether other plasma cholesterol pools (LDL and HDL_c) might also be intestinal products.

METHODS

Animals and diets

Adult, purebred foxhounds of both sexes were selected from one of two groups: a control group fed a mixed fat (lard–corn oil 1:1) diet in which 25% of the calories was contributed by the fats and with no added cholesterol; or a cholesterol-fed group which received a previously described diet (12) containing 42% of the calories as hydrogenated coconut oil and added cholesterol at a level of 15 mg/Cal. The animals were weighed and their plasma cholesterol and triglyceride concentrations determined weekly. They had consumed their diets for at least 2 months, and the plasma cholesterol levels had plateaued before the animals were selected for experiments.

Isotopes

[1-^{3}H]Retinol (all *trans*) was obtained from New England Nuclear (Boston, MA). Greater than 94% of the retinol radioactivity comigrated with purified retinol on silica gel H TLC plates developed in chloroform. [4-^{14}C]Cholesterol was obtained from Amersham Searle Corporation (Arlington Heights, IL); the radiopurity exceeded 97% when checked by TLC on silica gel H plates developed in hexane–diethyl ether 50:50.

Lymph collection

The procedure for creating a lymph fistula was a modification of that previously described by Rajpal and Kirkpatrick (13). Briefly, the animals were anesthetized with halothane, the left external jugular vein was ligated 1 cm inferior to its junction with the omobrachial vein, and the superficial cervical, cephalic, and subclavian veins were ligated at their junctions with the external jugular. The internal jugular was ligated at its junction with the brachiocephalic vein and the brachiocephalic was ligated just inferior to that junction. This created a blind pouch into which thoracic duct lymph flowed unrestricted, and thereby allowed us to sample thoracic duct lymph without performing a thorocotomy. A cannula, previously coated with TDMAC-heparin (Polysciences, Inc., Warrington, PA), was sutured into the isolated portion of the external jugular 1 cm cranial to the orifice of the thoracic duct and externalized.

The animals had recovered from the anesthetic and were standing erect within 1 hr after surgery. The isotopes were administered orally in a few ounces of canned dog food just prior to the animal's meal the following morning. Lymph was collected continuously thereafter in Fenwal blood packs (Travenol Laboratories, Inc., Deerfield, IL) affixed to the dog's abdomen and containing 300 mg of disodium EDTA in 20 ml (d 1.006 g/ml) of saline, pH 7.2. Lymph was usually collected for 4-hr periods and, in most instances, the 4 to 8-hr fraction was used to harvest the radiolabeled chylomicrons.

Chylomicron preparation

To prepare $S_f > 400$ chylomicrons, lymph was gently layered under a few milliliters of saline (d 1.006 g/ml) containing EDTA and centrifuged at 75,000 *g* for 20 min in an SW-41 rotor (Beckman Instrument Co., Palo Alto, CA). A thick creamy layer less than 2 cm from the tops of the tubes was removed by tube slicing, resuspended in the d 1.006 g/ml NaCl solution, and centrifuged again as described above. The washed $S_f > 400$ chylomicrons were suspended in the d 1.006 g/ml NaCl solution and used within 48 hr.

S_f 20–400 chylomicrons were prepared from the bottom fractions of the first centrifugation. Residual $S_f > 400$ particles were removed by centrifuging at 75,000 *g* for 30 min and slicing 2 cm from the top of the tube. The bottom fractions were then spun at 193,000 *g* for 16 hr. The tubes were sliced 2 cm from the top and the S_f 20–400 chylomicrons were removed, resuspended in the d 1.006 g/ml NaCl solution, and used within 72 hr.

Chylomicron administration

Chylomicrons (40–50 mg of triglyceride and 1–2.5 μCi of [1-^{3}H]retinol or [4-^{14}C]cholesterol per kilogram of body weight) were administered through the left external jugular vein. Blood samples were obtained from the right external jugular vein at intervals from 5 min to 8 hr after chylomicron administration.

In those studies in which liver biopsies were taken, the animals were anesthetized with pentobarbital, the liver was exposed through a vertical abdominal incision, and the left external jugular and left femoral veins were cannulated. Chylomicrons were

administered through the jugular cannula. Blood samples were obtained from the femoral cannula and liver biopsies were taken simultaneously.

Lipoprotein characterization

Blood obtained from the dogs was mixed with disodium EDTA (final concentration 1 mg/ml) and the plasma was separated from the cells by centrifugation. Care was taken when removing the plasma to recover any creamy layer floating on the surface. The plasma lipoproteins were separated sequentially by ultracentrifugation in the SW-41 rotor, essentially as described by Havel, Eder, and Bragdon (14) and with reference to the nomogram of Dole and Hamlin (15). Plasma lipoproteins were isolated by centrifuging as follows: $S_f > 400$, at 75,000 *g*, 30 min; S_f 20–400, 16 hr at a solvent density of 1.006 g/ml; d 1.006–1.020 and d 1.020–1.063 g/ml fractions, 18 hr at solvent densities of 1.020 and 1.063 g/ml, respectively; and d 1.063–1.21 g/ml, 44 hr at a solvent density of 1.21 g/ml. The $S_f < 400$ lipoproteins were centrifuged at 193,000 *g*.

The $S_f > 400$ lipoproteins were also isolated from the plasma of hypercholesterolemic dogs by density gradient centrifugation using the SW-41 rotor. The gradient was prepared by adding 3.0 ml of 1.006 g/ml NaCl solution to each tube and then successively underlayering with 3.0 ml each of KBr–NaCl solutions of density 1.019 and 1.04 g/ml, and 3.6 ml of plasma, previously adjusted to d 1.063 g/ml with solid KBr. The tubes were spun at 75,000 *g* for 15 min and lipoproteins of $S_f > 400$ were removed by slicing the tubes 2.0 cm from the top. Salt blank tubes run simultaneously were sliced at 1-cm intervals, and the density at each interval was determined so as to define the gradient. A linear gradient was produced in the top 6 cm of the tube, which ranged in density from 1.004 to 1.056 g/ml.

Geon-Pevikon preparative electrophoresis (16) was used to purify β-VLDL from the d < 1.006 g/ml fraction and to isolate LDL and HDL_c. Electrophoresis in 0.5% agarose was performed as described by Noble (17).

Liver and bile analysis

Liver biopsies, ranging from 100–200 mg (wet wt), were immediately rinsed with cold saline and weighed. The samples were placed in glass screw-cap vials and 5 ml of 6 N KOH in 33% ethanol was added. The vials were incubated at 37°C until the samples had been completely digested and then extracted three times with 5 ml of hexane. The hexane extracts were pooled in a scintillation vial and blown dry. The residue was dissolved in 0.5 ml of ethanol, 15 ml of Biofluor (New England Nuclear) was added, and the radioactivity was measured as described below. Aliquots of bile (1 ml) were saponified, extracted, and counted exactly as described for liver.

Lipid determinations

Lipoprotein cholesterol, triglyceride, and phospholipid concentrations were determined as described previously (3). Retinol was extracted from the plasma or individual lipoproteins into hexane after the addition of an equal volume of 95% ethanol. The free and the esterified retinol were separated by column chromatography on alumina exactly as described by Ross and Zilversmit (8) or by TLC on silica gel H plates developed in chloroform. Retinol or retinol palmitate standards, purified by high performance liquid chromatography (gifts from Dr. John Bieri, NIAMDD, NIH), were run simultaneously and the zones were identified under ultraviolet light.

Radioactivity determinations

The radioactivity present in the plasma lipoprotein fractions or liver extracts was measured by liquid scintillation counting in a Packard Model 3390 LSC (Packard Instruments). Counting efficiency was determined using [^{3}H]- or [^{14}C]toluene internal standards (Amersham/Searle) and all samples were counted to a 2 sigma error of 2%.

Calculations

The radioactivity remaining in the plasma compartment or liver after intravenous administration of labeled chylomicrons was expressed as a fraction of the activity administered. The plasma volume was assumed to be 4.5% of the body weight, a value we verified in three dogs, using the Evan's blue dye dilution method (18). The liver mass was determined by excising and weighing the liver immediately after the animals were killed. The liver blood volume was determined in one experiment using ^{51}Cr-labeled red cells, and the error in total liver activity due to plasma radioactivity contamination was found to be insignificant.

RESULTS

Effect of diet on plasma cholesterol concentration

Table 1 shows the fasting cholesterol concentration and its distribution among the lipoproteins in typical control and cholesterol-fed foxhounds. A detailed description of the changes that occur in plasma

TABLE 1. Cholesterol distribution among the plasma lipoproteins in typical control and hypercholesterolemic dogs

Recipient	Plasma Cholesterol Concentration	VLDL	β-VLDL	IDL	LDL	$HDL_{1(c)}$	HDL_2
	mg/dl			*% of the total pool*			
Control	161	11.2	N.D.[a]	1.2	11.8	13.3	72.0
Hypercholesterolemic	1189	3.3	18.1	27.8	24.5	14.4	12.0

[a] None detected.

cholesterol concentration and distribution as hypercholesterolemia progresses has been reported previously by Mahley et al. (1, 12). In the present study a diet rich in saturated and short-chain fatty acids (coconut oil) and containing high levels of cholesterol (5% by weight) was used to induce hypercholesterolemia in euthyroid animals. The changes that occurred were qualitatively and quantitatively similar to those previously described (1, 12). The plasma cholesterol of the animals began to increase within days after the initiation of the diet and by 6 weeks had plateaued at between 750 and 1200 mg/dl. At the intermediate cholesterol concentrations (200–750 mg/dl), greater than 90% of the increase could be accounted for by elevation in the LDL and HDL_c cholesterol concentrations, although increases in the amount of $d < 1.006$ g/ml cholesterol were also detected. As the plasma cholesterol concentration exceeded 750 mg/dl, the cholesterol associated with particles of $d < 1.006$ g/ml became a major fraction of the plasma cholesterol pool, accounting (in some instances) for as much as 30% of the total pool.

When the $d < 1.006$ g/ml lipoproteins from fasted hypercholesterolemic animals were subfractionated by preparative ultracentrifugation, those of $S_f > 400$ accounted for 13–50% of the total $d < 1.006$ g/ml cholesterol. As shown in **Fig. 1,** these particles exhibited β-mobility during agarose electrophoresis. The S_f 20–400 fraction contained both β- and pre-β-migrating bands; the latter band, however, was a minor component (Fig. 1).

β-VLDL, nondetectable in control animals, was the carrier of 80–100% of the cholesterol in the $d < 1.006$ g/ml fractions of hypercholesterolemic recipients (Fig. 1, Table 1) (1, 12) and accounted for 80–100% of the total $d < 1.006$ g/ml lipoproteins. β-VLDL averaged 90–95% by weight lipid with an average molar ratio of 79% cholesterol, 3.5% triglyceride, and 17.5% phospholipid. The cholesterol was 85–90% esterified.

Of the remaining plasma cholesterol pool, an average of 85% was contained in the d 1.006–1.063 g/ml lipoprotein fraction. The distribution of the d 1.006–1.063 g/ml cholesterol pool between LDL and HDL_c varied as the total plasma cholesterol concentration changed, but the distribution shown in Table 1 is typical of that seen in animals with a total plasma cholesterol exceeding 1000 mg/dl.

Plasma distribution of tritiated retinol after oral administration

Fig. 2 shows typical changes in the radioactivity in the plasma lipoproteins at various times after administration of a tritiated retinol-containing meal to a hypercholesterolemic foxhound. All animals tested (two control, two hypercholesterolemic) showed similar responses. The initial rise in activity was confined exclusively to the $d < 1.006$ g/ml lipoprotein fraction and, presumably, represented labeled chylomicrons and chylomicron remnants. The $d < 1.006$ g/ml activity began to drop within 6–10 hr after the meal in both control and hypercholesterolemic animals. However, in every instance, before the $d < 1.006$ g/ml lipoprotein activity had returned to baseline levels, a second rise in the $d < 1.006$ g/ml activity occurred. This rebound peaked 7–10

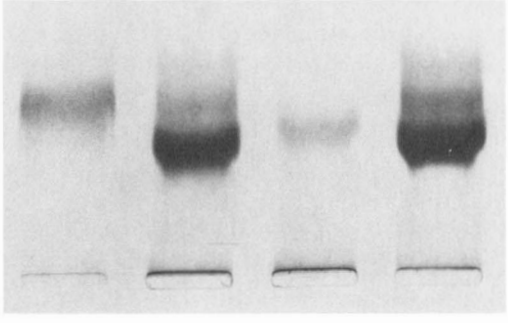

Fig. 1. The electrophoretic mobility of the $d < 1.006$ g/ml lipoprotein fractions from control and hypercholesterolemic dogs fasted for 16 hr. Left to right: $d < 1.006$ g/ml fraction from a control dog; $d < 1.006$ g/ml fraction from a hypercholesterolemic dog; lipoproteins of $S_f > 400$ from the hypercholesterolemic dog; and S_f 20–400 fraction from the hypercholesterolemic dog. The $S_f > 400$ fraction was isolated by density gradient ultracentrifugation as described in Methods.

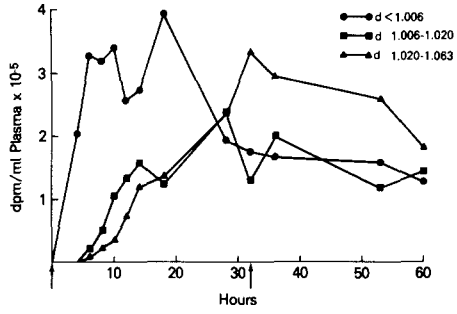

Fig. 2. Radioactivity distribution among three density fractions of the plasma from a hypercholesterolemic dog fed 2 mCi of [1-^{3}H]retinol at time t = 0. (● —— ●), d < 1.006 g/ml fraction; (■ —— ■), d 1.006–1.020 g/ml fraction; (▲ —— ▲), d 1.020–1.063 g/ml fraction. Arrows indicate times at which the animal received its high cholesterol meals. Less than 4% of the plasma radioactivity was contained in the d > 1.063 g/ml fraction at any time point, and at 18 and 28 hr, greater than 94% of the d 1.020–1.063 g/ml radioactivity was associated with β-migrating lipoproteins.

hr after the original peak and closely coincided with (in controls) or slightly preceded (in hypercholesterolemic animals) a rise in the d 1.006–1.063 g/ml lipoprotein radioactivity. More than 94% of the d 1.020–1.063 g/ml radioactivity was associated with the β-migrating LDL; i.e., less than 6% of the activity was associated with the α_2-migrating HDL_c. Greater than 90% comigrated with retinol palmitate during TLC.

The major difference between control and cholesterol-fed animals was the duration in the plasma of the retinyl ester activity associated with LDL. In controls, the LDL activity had dropped to half of the peak value within 15 hr, whereas in the hypercholesterolemic animals, the LDL activity was still 70% of its peak activity after 24 hr.

Secretion and distribution of retinol in lymph lipoproteins

To determine whether the intestine incorporated retinol into the d 1.006–1.063 g/ml lipoprotein fraction, we collected lymph from a hypercholesterolemic dog fed [1-^{3}H]retinol. **Fig. 3** shows that at no time within the first 20 hr after oral administration of the labeled retinol was there significant incorporation of retinol label into the d 1.006–1.063 g/ml ($S_f < 20$) lipoprotein fraction of lymph. This figure further shows that insignificant quantities of radioactivity were being secreted in the lymph at the time (16 hr) at which the radioactivity associated with the d 1.006–1.063 g/ml fraction was rapidly increasing in the plasma. Therefore, it appears that the plasma radioactivity associated with d 1.006–1.063 g/ml lipoproteins in the dog receiving retinol orally (Fig. 2) was not a product of the intestine.

Turnover of intravenously administered lymph chylomicrons in control and hypercholesterolemic dogs

Lymph chylomicrons of $S_f > 400$ from normal and cholesterol-fed dogs were injected intravenously into normal and hypercholesterolemic recipient dogs. The lipid composition of the lymph chylomicrons is shown in **Table 2.** An average (for all studies) of 93.3 ± 0.014 (SEM)% of the [1-^{3}H]retinol and 86.2 ± 0.048% of the [4-^{14}C]cholesterol in the intravenously administered lymph fractions was esterified. The chylomicrons from cholesterol-fed donors contained slightly more cholesterol per particle than chylomicrons from normal dogs. The S_f 20–400 particles were more enriched in cholesterol and phospholipids than were the $S_f > 400$ lipoproteins. There were no demonstrable differences in the rate at which lymph $S_f > 400$ chylomicrons from normal

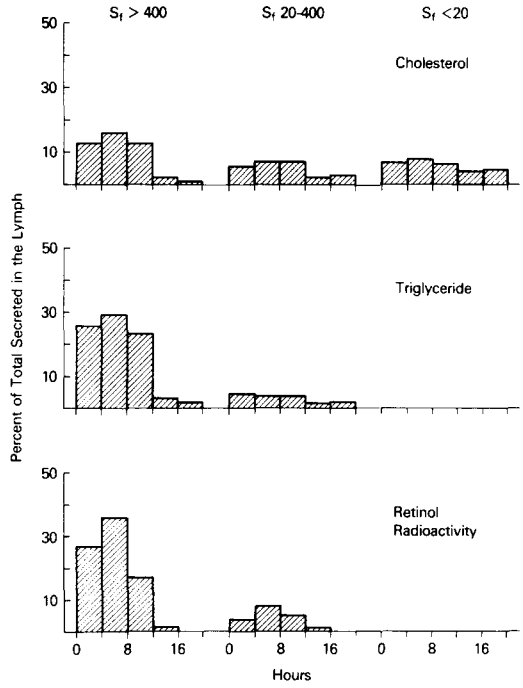

Fig. 3. The distribution of retinol radioactivity and mass of cholesterol and triglyceride among three density fractions of thoracic duct lymph at several times after [1-^{3}H]retinol was administered in a high cholesterol meal to a hypercholesterolemic dog. Each bar represents the quantity of cholesterol, triglyceride, or radioactivity recovered in a 4-hr interval divided by the total quantity of that component recovered after 20 hr of continuous collection. Small quantities of triglycerides and [1-^{3}H]retinol radioactivity were recovered in the $S_f < 20$ lymph fraction, but the recovery at each time interval represented less than 0.2% of the total of each compound.

TABLE 2. Lipid composition of chylomicrons used for injection

	N	Particle Size (S_f)	Molar Ratio		
			Cholesterol	Triglyceride	Phospholipid
Cholesterol-fed	3	>400	10.7 (1.4)[a]	83.1 (2.1)	6.2 (0.6)
	3	20–400	18.4 (2.2)	64.6 (4.1)	16.1 (2.5)
Control	2	>400	2.3 (0.4)	94.7 (1.2)	3.0 (1.6)

[a] Mean (SEM).

or cholesterol-fed animals was cleared from the plasma of recipients; therefore, the results obtained utilizing chylomicrons from normal and cholesterol-fed dogs were pooled for statistical analyses.

Fig. 4 shows the pooled data describing plasma clearance of $S_f > 400$ chylomicrons from five control and seven hypercholesterolemic recipients. Each recipient had been fasted for 16 hr before injection of the $S_f > 400$ chylomicrons (doubly labeled with [1-^{3}H]retinol and [4-^{14}C]cholesterol). In every instance, there was an immediate rapid drop in plasma retinol radioactivity after injection. The $t_{1/2}$ for the initial clearance in fasted animals ranged from 3 to 10 min in five control recipients (Fig. 4A) and 3 to 8 min in seven hypercholesterolemic recipients (Fig. 4B). The initial rapid clearance accounted for the removal of 80–90% of the injected activity. There was always a slight rebound of retinol radioactivity in both controls and hypercholesterolemic recipients, but the total plasma retinol radioactivity never exceeded 12% of the administered activity in either group after 2 hr. In no instance was there any indication that hypercholesterolemic animals had an impaired capacity to clear chylomicrons or chylomicron remnants from the plasma compartment.

The plasma clearance of cholesterol radioactivity was similar to that of retinol. In the hypercholesterolemic animals a slightly larger fraction of the cholesterol radioactivity was retained in the plasma compartment (Fig. 4B); however, after the initial rapid clearance, the activity reappearing in the plasma never exceeded 16% of the administered activity and this activity probably resulted from exchange of the labeled cholesterol among other plasma lipoproteins.

When S_f 20–400 chylomicrons were injected into hypercholesterolemic recipients, the results were similar to those obtained with the $S_f > 400$ particles (data not shown). The only consistent difference noted was that the $t_{1/2}$ for plasma clearance of the S_f 20–400 chylomicrons was slightly longer (13–15 min in two hypercholesterolemic recipients and 11 min in a control recipient). Again, there was no evidence that clearance of S_f 20–400 chylomicrons from the plasma was significantly retarded in the hypercholesterolemic animals.

Similar studies were performed in nonfasted dogs to determine if there was a delayed clearance of retinol labeled $S_f > 400$ chylomicrons. The results obtained in a control dog (Fig. 4A inset) and in a hypercholesterolemic dog (Fig. 4B inset) were compared. Both animals received an intravenous injection 4 hr after they had consumed their normal or cholesterol-rich meal. The plasma clearance rates were not significantly different from those obtained in the fasted animals.

To determine if there were any retinol-labeled lipoproteins of intestinal origin that were inefficiently cleared from the plasma of hypercholesterolemic animals, freshly collected whole lymph was given intravenously to a hypercholesterolemic dog. The clearance rates of retinol-labeled whole lymph and of

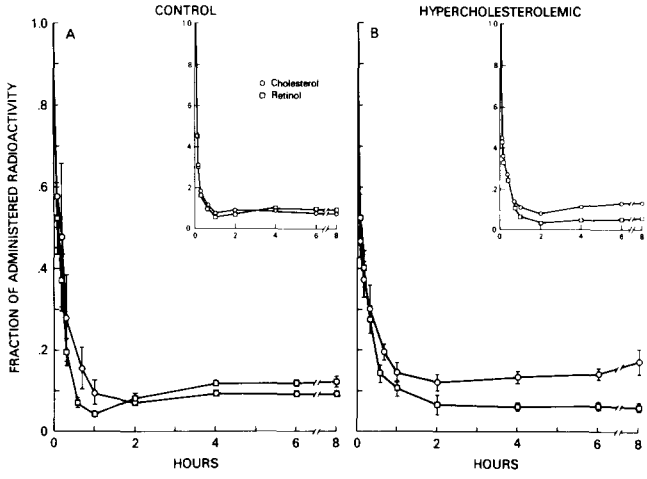

Fig. 4. The fraction of administered radioactivity remaining in the plasma at several times after intravenous administration of $S_f > 400$ chylomicrons doubly labeled with [1-^{3}H]retinol (□) and [4-^{14}C]cholesterol (○) to control (A) and hypercholesterolemic (B) dogs previously subjected to a 16-hr fast. The mean ± SEM (bar) value for five control and seven hypercholesterolemic recipients is shown. The inset shows the clearance of doubly labeled chylomicrons injected 4 hr after a meal into one control (A) and one hypercholesterolemic (B) recipient.

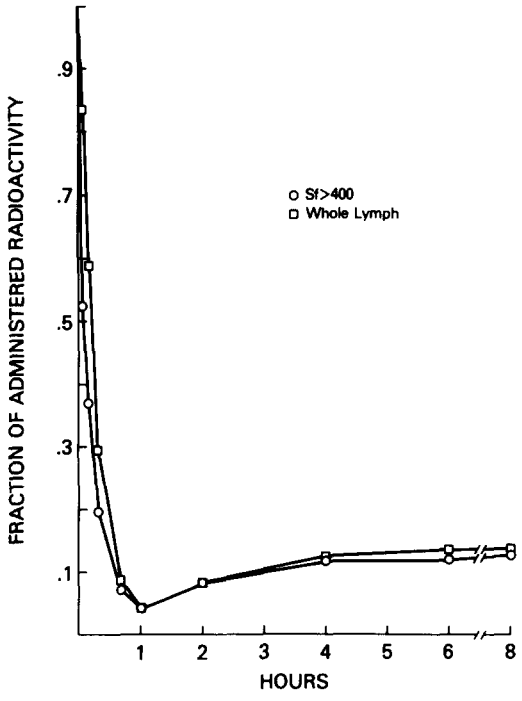

Fig. 5. The fraction of the administered radioactivity remaining in the plasma at several times after intravenous administration of $S_f > 400$ chylomicrons (○) and whole lymph (□) to a hypercholesterolemic recipient. Both the $S_f > 400$ chylomicrons and the whole lymph were labeled with [1-^{3}H]retinol. The lymph was injected approximately 1 month after the $S_f > 400$ chylomicrons and no residual radioactivity from the previous injection was detectable.

$S_f > 400$ chylomicrons in the same animal are compared in **Fig. 5.** The early, rapid clearance of retinol-labeled whole lymph lipoproteins was slightly slower than that of the $S_f > 400$ lipoproteins. The slower rate was likely due to the presence of S_f 20–400 particles that were cleared slightly more slowly than $S_f > 400$. However, the two curves (isolated chylomicrons versus whole lymph) are not significantly different. This supports the contention that clearance of chylomicrons or chylomicron remnants is not defective in hypercholesterolemic dogs. Furthermore, this study suggested that the isolation of the lymph chylomicrons by ultracentrifugation had not significantly altered their metabolic behavior.

Liver uptake of chylomicron remnants

The chylomicron remnants generated by hydrolysis of chylomicron triglycerides have been shown to be taken up by the liver (19). The plasma clearance and liver uptake of [1-^{3}H]retinol after intravenous injection of labeled $S_f > 400$ chylomicrons into control and hypercholesterolemic dogs are compared in **Fig. 6,** and the distributions of [1-^{3}H]retinol in various lipoprotein fractions are presented in **Table 3.**

The retinol label was rapidly cleared from the $S_f > 400$ fraction of both normal and hypercholesterolemic recipients (Table 3), and in every instance there was a transient rise and fall in the S_f 20–400 radioactivity. Little radioactivity was recovered in the lipoproteins of $S_f < 20$ within 60 min after isotope administration, although increases of varying mag-

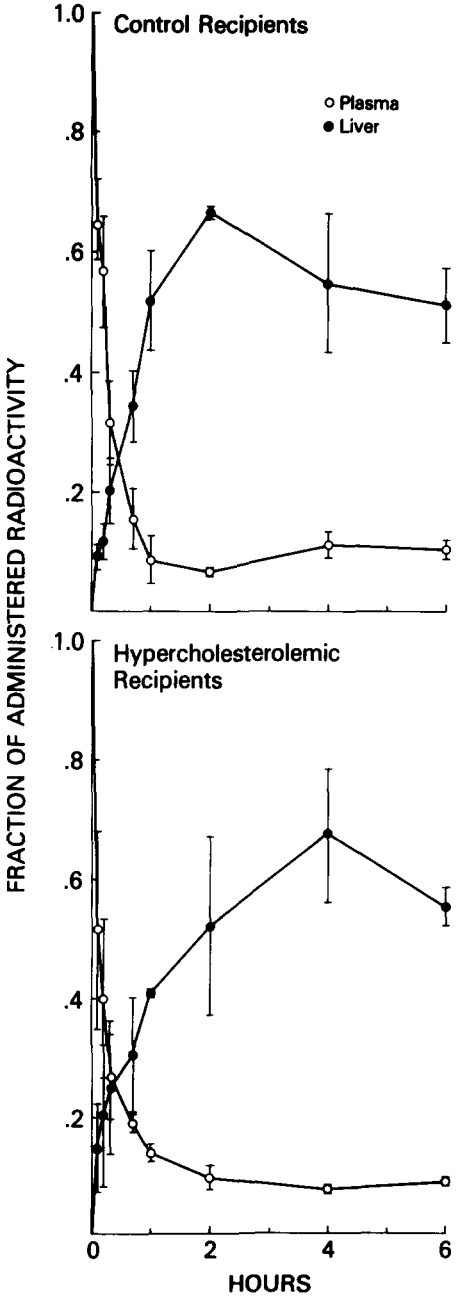

Fig. 6. The fraction of the administered radioactivity remaining in the plasma (○) and contained in the liver (●) at various times after intravenous administration of [1-^{3}H]retinol-labeled $S_f > 400$ chylomicrons. Each point represents the mean ± half the range (bars) values for two control (upper panel) and two hypercholesterolemic (lower panel) recipients.

TABLE 3. Distribution of the plasma [1-^{3}H]retinol radioactivity among three density fractions at several times after $S_f > 400$ chylomicron administration to control and hypercholesterolemic foxhounds

	S_f	Minutes						
		5	10	20	40	60	120	240
		%[a]						
Control (n = 2)	>400	57.4 (0.7)[b]	49.0 (3.0)	26.5 (2.8)	13.7 (1.2)	4.8 (3.6)	1.3 (0.3)	2.2 (0.1)
	20–400	16.8 (5.3)	5.7 (4.6)	3.4 (2.9)	2.3 (0.8)	2.1 (0.4)	1.4 (0.3)	2.3 (2.1)
	<20	1.0 (0.5)	2.1 (1.7)	1.7 (1.3)	1.7 (1.0)	1.8 (0.8)	4.0 (0.5)	8.8 (0.1)
Hypercholesterolemic (n = 2)	>400	44.6 (14.4)	31.4 (10.2)	19.8 (6.2)	13.4 (2.4)	9.3 (0.9)	6.4 (1.2)	3.5 (2.4)
	20–400	6.4 (2.4)	7.5 (2.0)	6.6 (0.9)	5.2 (0.9)	4.4 (2.1)	2.7 (1.0)	2.6 (1.3)
	<20	0.5 (0.1)	1.3 (0.9)	0.5 (0.1)	0.6 (0.1)	0.5 (0.2)	0.8 (0.1)	2.0 (0.6)

[a] Percentage of administered activity remaining in the plasma compartment.
[b] Range/2.

nitudes were observed in all animals between 2 and 6 hr after administration. The majority of the radioactivity in the $S_f < 20$ fraction was associated with LDL.

The rapid clearance of the chylomicrons in both the control and cholesterol-fed dogs was followed by the appearance of the label in the liver (Fig. 6). The activity peaked in the control livers at 2 hr and in the livers of the hypercholesterolemic dogs at 4 hr. However, in both cases the mean activity in the livers at peak uptake was 68% of the injected dose of [1-^{3}H]retinol-labeled chylomicrons. The activity in the liver began to decrease after the peak. Less than 2% of the administered retinol radioactivity could be detected in the saponifiable fraction of bile.

Three of the recipient dogs used in these studies (two hypercholesterolemic and one control) received chylomicrons doubly labeled with [4-^{14}C]cholesterol and [1-^{3}H]retinol. The curves describing liver uptake of [4-^{14}C]cholesterol radioactivity were virtually identical to those of [1-^{3}H]retinol radioactivity. In each instance the fraction of the administered cholesterol label appearing in the liver in a given interval exceeded that of administered retinol; however, the difference in total uptake of the two labels at any time never exceeded 6%.

In addition to the use of tracer doses of [1-^{3}H]-retinol- and [4-^{14}C]cholesterol-labeled lymph lipoproteins, studies were designed to infuse large quantities of doubly labeled lymph lipoproteins as might occur with the consumption of a high fat-high cholesterol meal. A hypercholesterolemic dog was injected intravenously with 240 ml of lymph (7.9 g of triglyceride) divided into equal doses and given at 15-min intervals over a 2-hr period. Plasma samples and lipoprotein fractions were analyzed at intervals from 30 min to 72 hr after the final injection of the lymph. The distribution of the [1-^{3}H]retinol radioactivity in the lymph lipoproteins is given in **Table 4.** Approximately 90% of the administered retinol had been removed from the plasma within 30 min after injection (Table 4). The retinol-labeled lipoproteins of $S_f > 400$ decreased rapidly, whereas the S_f 20–400 lipoproteins, which decreased initially, increased slightly between 3 and 6 hr, the time when liver uptake would have been expected to peak (Fig. 3). Furthermore, the retinol activity in the d 1.020–1.063 g/ml fraction increased progressively; there was essentially no activity at 30 min, but after 22 hr the d 1.020–1.063 g/ml became the principal lipoprotein with activity. This represented an absolute increase in radioactivity of the d 1.020–1.063 g/ml fraction, beyond what was administered. The retinol labeled d 1.020–1.063 g/ml and d 1.063–1.21 g/ml lipoproteins may result from catabolism of the $d < 1.006$ g/ml particles or incorporation of label into newly synthesized lipoproteins (see Discussion). The clearance of the cholesterol label, in general, paralleled the results obtained with retinol.

DISCUSSION

This study was undertaken to determine to what extent chylomicron remnant cholesteryl esters con-

TABLE 4. Distribution of [1-^{3}H]retinol radioactivity among six density fractions of plasma at several times after lymph was administered intravenously in divided doses[a] to a hypercholesterolemic dog

Density Fraction[b]	Lymph[c]	Hours										
		.5	1	2	3	4	6	8	11	22	48	72
		%[d]										
$S_f > 400$	79.2	6.9	5.6	4.4	2.6	1.9	1.9	1.8	1.4	0.7	1.2	6.6
S_f 20–400	20.2	3.1	3.1	3.9	5.0	5.2	5.3	5.4	6.7	3.2	0.3	1.2
d 1.006–1.020	0.3	0.3	0.4	1.1	1.6	2.4	3.9	4.1	3.9	2.0	2.5	2.4
d 1.020–1.063	0.4	0.4	0.5	0.7	1.0	1.2	2.0	4.1	5.9	13.5	9.6	6.8
d 1.063–1.210	0.3	0.3	0.3	0.5	0.5	0.5	0.6	0.7	0.8	1.2	2.5	1.9

[a] Thirty ml of lymph was injected intravenously at 15-min intervals until 240 ml of lymph had been administered. The first blood sample was taken 30 min after the last injection.
[b] The d > 1.21 g/ml fraction (not shown) never contained more than 0.4% of the administered [1-^{3}H]retinol.
[c] Radioactivity distribution in the administered lymph.
[d] Percentage of administered radioactivity remaining in the plasma compartment.

tribute to the hypercholesterolemia in cholesterol-fed foxhounds, and which of the lipoprotein fractions might be immediate products of chylomicron metabolism. We utilized the approach of Ross and Zilversmit (8), in which retinol-labeled chylomicrons were obtained from the thoracic lymph duct of donor animals and administered to normal and hypercholesterolemic recipients, and the plasma clearance and liver uptake of these chylomicrons were followed. This approach assumes that esterified retinol contained in the lymph chylomicrons is not removed during triglyceride hydrolysis in the periphery, but returns to the circulation with the chylomicron remnants. It is then cleared with the remnants from the circulation by the liver and, under normal conditions, the retinol reappears in the plasma only in the unesterified form associated with retinol-binding protein. The hydrated density of retinol-binding protein exceeds 1.21 g/ml (20), and therefore any plasma lipoproteins containing esterified retinol are presumed to be of intestinal origin.

We used foxhounds in this study because they develop a marked hypercholesterolemia and because the changes that occur in their lipoproteins with increasing plasma cholesterol concentration have been well characterized (1, 12). We induced hypercholesterolemia using a diet rich in coconut oil and cholesterol. Cholesterol accumulated in the plasma in three distinct lipoprotein fractions: β-VLDL, LDL, and HDL_c (1). The d < 1.006 g/ml fraction of hypercholesterolemic dogs with plasma cholesterol levels in excess of 750 mg/dl contained predominantly the β-VLDL. β-VLDL is not normally detectable in the plasma of foxhounds but carries increasing proportions of the plasma cholesterol pool as the plasma cholesterol concentration increases. The hypercholesterolemic recipients used in this study carried between 15 and 30% of the plasma cholesterol in the β-VLDL fraction. At the present time, the origin of the β-VLDL and the HDL_c is unknown.

When control and hypercholesterolemic foxhounds are fed meals containing [1-^{3}H]retinol, there is an initial rise and fall in the plasma d < 1.006 g/ml radioactivity, presumably due to radiolabeled chylomicrons and chylomicron remnants. This is followed 6–10 hr later by a second rise in plasma activity, a major portion of which is contained in lipoproteins of d 1.006–1.020 and d 1.020–1.063 g/ml. More than 90% of the retinol in this fraction is esterified. Analysis of the distribution of [1-^{3}H]retinol radioactivity in the lipoproteins of the thoracic duct lymph following oral administration of the retinol indicated that the labeled d 1.006–1.063 g/ml lipoproteins did not originate in the intestine, and an absolute increase in concentration of these lipoproteins was evident following the intravenous injection of chylomicrons or whole lymph.

Two possibilities remain to explain the appearance of label in the d 1.006–1.063 g/ml lipoproteins. It is possible that some chylomicrons are sequestered in the periphery for extended periods and catabolized to lipoproteins of d 1.006–1.063 g/ml that are not rapidly cleared by the liver. Alternatively, the liver may incorporate retinyl esters into some hepatic lipoproteins. As to the latter possibility, it should be noted that the disappearance of label from the liver generally coincided with the appearance of radioactivity in the d 1.020–1.063 g/ml fraction. Because of this, caution must be exercised before equating the appearance of retinol in lipoproteins with intestinal synthesis of those lipoproteins.

A major conclusion of these studies is that canine hypercholesterolemia does not result from impaired hepatic clearance of chylomi-

crons doubly labeled with [4-^{14}C]cholesterol and [1-^{3}H]retinol were rapidly cleared from the plasma of both normal and hypercholesterolemic dogs, and a major fraction of the administered radioactivity appeared in the liver shortly thereafter (Fig. 6). Thus, even in animals with very severe hypercholesterolemia, the liver was capable of rapidly removing the chylomicron remnants from the circulation. It seems unlikely, based on the rate at which intravenously administered radioactivity appeared in the liver of hypercholesterolemic recipients, that the remnants produced within the first 4–6 hr equilibrated with any sizeable plasma cholesterol pools prior to liver uptake. For example, the average size of the β-VLDL cholesterol pool was 2.6 g in hypercholesterolemic dogs. If this represents chylomicron remnant cholesterol, thus indicating that the labeled cholesterol in the liver had equilibrated with the β-VLDL cholesterol pool prior to hepatic uptake, then the minimum rate of chylomicron cholesterol production would have to exceed 15 g/day to maintain the β-VLDL cholesterol pool in a steady state.[2] In no instance was such a high rate of secretion measured in the thoracic duct lymph. When thoracic duct lymph was collected continuously for 24 hr from two hypercholesterolemic donors fed high-cholesterol meals, less than 6 g of cholesterol was recovered in each instance. Although this is not a precise method of measuring cholesterol absorption in vivo, it does indicate that the rate at which intestinal cholesterol enters the plasma compartment is considerably less than the 15 g/day that would be required to maintain the β-VLDL pool in a steady state if that pool were derived solely from chylomicron remnants.

This does not rule out the possibility that the β-VLDL is heterogeneous and that a subfraction of this pool with a rapid turnover represents chylomicron remnants. However, it is clear that the β-VLDL cholesterol pool is not a significant barrier to liver uptake of cholesteryl esters of remnants produced within the first few hours after chylomicron administration.

The same argument can be applied to the LDL and HDL_c cholesterol pools. The LDL pool represents the largest plasma pool of cholesterol in hypercholesterolemic dogs, and less than 6% of the administered retinol radioactivity could be detected in the LDL within 6 hr after chylomicron administration. Furthermore, the turnover of LDL is far too slow in hypercholesterolemic animals to account for the rapid uptake of cholesterol and retinol-labeled remnants by the liver (21). As for HDL_c, at no time after oral administration of [1-^{3}H]retinol to normal or hypercholesterolemic recipients, nor after intravenous administration of any [1-^{3}H]retinol-labeled lymph fraction was more than 4% of the administered retinol radioactivity associated with HDL_c. Thus we conclude that the β-VLDL, LDL, and HDL_c cholesterol pools do not represent chylomicron remnant cholesterol trapped in the plasma compartment by a saturated, hepatic remnant clearance mechanism.

The dog is strikingly different from the rabbit in this respect. Rabbits have long been known to develop an extreme hypercholesterolemia when challenged with dietary cholesterol, and a major portion of this increase in cholesterol concentration is confined to the $d < 1.006$ g/ml fraction (22). Ross and Zilversmit (8) have now presented compelling evidence that the increased cholesterol concentration of this lipoprotein fraction is due principally to the accumulation of cholesteryl ester-rich chylomicron remnants.

These conclusions about the origin of the $d < 1.006$ g/ml cholesterol pool may differ from those of Ross and Zilversmit (8) because different animal models of hypercholesterolemia were used in the two studies; that is to say, the defects causing the plasma cholesterol to increase in the $d < 1.006$ g/ml fraction are different in the dog and the rabbit. Cholesterol is not a normal constituent of the rabbit's diet. The rabbit's principal source of cholesterol is synthesis; thus, it is not surprising that this animal has failed to develop an efficient mechanism for excretion of excess dietary cholesterol. By contrast, dogs are carnivorous and have evolved with an extremely efficient mechanism for maintaining cholesterol homeostasis. It is virtually impossible to induce hyper-

[2] If it is assumed that all of the cholesterol label entering the liver equilibrates with the β-VLDL cholesterol pool prior to hepatic uptake, then a theoretical rate of β-VLDL cholesterol flux into the liver can be calculated from the equation:

$$\beta\text{-VLDL cholesterol entering the liver (mg/hr)} = \frac{\text{Change in liver radioactivity (dpm)}}{\beta\text{-VLDL cholesterol specific activity (dpm/mg)}}$$

This rate was calculated utilizing data from the same hypercholesterolemic recipients used in Fig. 6. The change in total liver radioactivity in the interval between 1 and 4 hr after intravenous administration of the cholesterol label (Fig. 6) was used as the numerator of the equation, because greater than 90% of the ^{14}C-labeled $S_f > 400$ particles had been cleared from the plasma compartment by 1 hr; thus, radioactivity entering the liver thereafter probably represented exclusively chylomicron remnants. The β-VLDL cholesterol specific radioactivity continuously decreased during the 1 to 4 hr interval in both recipients; however, the area under the specific radioactivity versus time curve was determined by planimetry, and the mean specific radioactivity in the 1 to 4 hr interval was calculated by dividing the area under that portion of the curve by ΔT (3 hr). The estimates of cholesterol flux so obtained represent minimum estimates based on the assumptions: *1*) that no loss of [4-^{14}C]cholesterol from the liver occurred during that interval, and *2*) that exchange of labeled cholesterol into the β-VLDL pool from other lipoproteins was minimal.

cholesterolemia in dogs simply by adding cholesterol to the diet. The two most widely used methods involve rendering the dog hypothyroid or feeding an essential fatty acid deficient diet. In both instances cholesterol must be added to the diet. The mechanism(s) by which these two procedures produce hypercholesterolemia is not known, although current evidence (23) suggests that they both inhibit the animal's ability to excrete endogenous cholesterol.

Our data are compatible with this concept and further suggest that the defect resulting in hypercholesterolemia in coconut oil-fed dogs involves not the ability of the animal's system to transport newly absorbed cholesterol to the hepatocyte, but rather the ability of the hepatocyte to excrete this cholesterol in the bile. It is reasonable to speculate that as the hepatic cholesterol pool expands, cholesteryl ester-rich lipoproteins are secreted into the plasma compartment. Evidence from recent studies in hypercholesterolemic rats (9–11) supports the contention that, in response to cholesterol feeding, the liver secretes cholesteryl ester-rich particles which may be equivalent to β-VLDL and LDL. It remains to be determined whether this is the case in dogs and, if so, whether the lipoproteins that accumulate (β-VLDL and LDL) represent nascent hepatic lipoproteins or end products of peripheral metabolism (hepatic remnants).

An alternate source of cholesteryl ester-rich lipoproteins in the plasma may be the peripheral tissues. Circulating lipoproteins may become enriched in cholesteryl esters as a result of direct transfer of cholesterol from tissue deposits. For example, it has been postulated that HDL_c is formed by overloading typical HDL with cholesterol from peripheral tissues (24). A similar mechanism could be responsible for the formation of cholesteryl ester rich β-VLDL and LDL.

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REFERENCES

1. Mahley, R. W., K. H. Weisgraber, and T. Innerarity. 1974. Canine lipoproteins and atherosclerosis. II. Characterization of the plasma lipoproteins associated with atherogenic and nonatherogenic hyperlipidemia. *Circ. Res.* **35:** 722–733.
2. Mahley, R. W., K. H. Weisgraber, T. Innerarity, H. B. Brewer, Jr., and G. Assmann. 1975. Swine lipoproteins and atherosclerosis. Changes in the plasma lipoproteins and apoproteins induced by cholesterol feeding. *Biochemistry.* **14:** 2817–2823.
3. Mahley, R. W., and K. S. Holcombe. 1977. Alterations of the plasma lipoproteins and apoproteins following cholesterol feeding in the rat. *J. Lipid Res.* **18:** 314–324.
4. Mahley, R. W., K. H. Weisgraber, and T. Innerarity. 1976. Atherogenic hyperlipoproteinemia induced by cholesterol feeding in the Patas monkey. *Biochemistry.* **15:** 2979–2985.
5. Rudel, L. L., R. Shah, and D. G. Greene. 1979. Study of the atherogenic dyslipoproteinemia induced by dietary cholesterol in rhesus monkeys (*Macaca mulatta*). *J. Lipid Res.* **20:** 55–65.
6. Goldstein, J. L., Y. K. Ho, M. S. Brown, T. L. Innerarity, and R. W. Mahley. 1980. Cholesteryl ester accumulation in macrophages resulting from receptor-mediated uptake and degradation of hypercholesterolemic canine β-very low density lipoproteins. *J. Biol. Chem.* **255:** 1839–1848.
7. Mahley, R. W., T. L. Innerarity, M. S. Brown, Y. K. Ho, and J. L. Goldstein. Cholesteryl ester synthesis in macrophages: stimulation by β-very low density lipoproteins from cholesterol-fed animals of several species. *J. Lipid Res.* **21:** 970–980.
8. Ross, A. C., and D. B. Zilversmit. 1977. Chylomicron remnant cholesteryl esters as the major constituent of very low density lipoproteins in plasma of cholesterol-fed rabbits. *J. Lipid Res.* **18:** 169–181.
9. Kris-Etherton, P. M., and A. D. Cooper. 1980. Studies on the etiology of the hyperlipemia in rats fed an atherogenic diet. *J. Lipid Res.* **21:** 435–442.
10. Swift, L. L., N. R. Manowitz, G. D. Dunn, and V. S. LeQuire. 1980. Isolation and characterization of hepatic Golgi lipoproteins from hypercholesterolemic rats. *J. Clin. Invest.* **66:** 415–425.
11. Noel, S. P., L. Wong, P. J. Dolphin, L. Dory, and D. Rubinstein. 1979. Secretion of cholesterol-rich lipoproteins by perfused livers of hypercholesterolemic rats. *J. Clin. Invest.* **64:** 674–683.
12. Mahley, R. W., T. L. Innerarity, K. H. Weisgraber, and D. L. Fry. 1977. Canine hyperlipoproteinemia and atherosclerosis. Accumulation of lipid by aortic medial cells in vivo and in vitro. *Am. J. Path.* **87:** 205–226.
13. Rajpal, S. G., and J. R. Kirkpatrick. 1972. Creation of a thoracic duct fistula: an improved technique. *J. Surgical Res.* **13:** 260–261.
14. Havel, R.J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
15. Dole, V. P., and J. T. Hamlin III. 1962. Particulate fat in lymph and blood. *Physiol. Rev.* **42:** 674–701.
16. Mahley, R. W., and K. H. Weisgraber. 1974. Canine lipoproteins and atherosclerosis. I. Isolation and characterization of plasma lipoproteins from control dogs. *Circ. Res.* **35:** 713–721.
17. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.

18. Gregersen, M. I., and R. A. Rawson. 1959. Blood volume. *Physiol. Rev.* **39:** 307–334.
19. Redgrave, T. G. 1970. Formation of cholesterol ester-rich particulate lipid during metabolism of chylomicrons. *J. Clin. Invest.* **49:** 465–471.
20. Kanai, M., A. Raz, and D. S. Goodman. 1968. Retinol-binding protein: the transport protein for vitamin A in human plasma. *J. Clin. Invest.* **47:** 2025–2044.
21. Mahley, R. W., T. L. Innerarity, K. H. Weisgraber, and S. Y. Oh. 1979. Altered metabolism (in vivo and in vitro) of plasma lipoproteins after selective chemical modification of lysine residues of the apoproteins. *J. Clin. Invest.* **64:** 743–750.
22. Gofman, J. W., F. Lindgren, H. Elliot, W. Mantz, J. Hewitt, B. Strisower, and V. Herring. 1950. The role of lipids and lipoproteins in atherosclerosis. *Science.* **111:** 166–171.
23. Pertsmilidis, D., E. H. Kirchman, and E.H. Ahrens, Jr. 1973. Regulation of cholesterol metabolism in the dog. 1. Effects of complete bile diversion and of cholesterol feeding on absorption, synthesis, accumulation, and excretion rates measured during life. *J. Clin. Invest.* **52:** 2353–2367.
24. Mahley, R. W. 1978. Alterations in plasma lipoproteins induced by cholesterol feeding in animals including man. *In* Disturbances in Lipid and Lipoprotein Metabolism. J. M. Dietschy, A. M. Gotto, Jr., and J. A. Ontko, editors. American Physiological Society, Bethesda, MD. 181–197.