Cholesterol absorption and turnover in hypercholesterolemic dogs

George W. Melchior[1] **and James F. Harwell***

Department of Physiology, Louisiana State University Medical Center, 1542 Tulane Avenue, New Orleans, LA 70112-2822, and Comparative Medicine Unit, Veterinary Resources Branch, National Institutes of Health, Bethesda, MD 20205*

Abstract Cholesterol absorption was measured in chronically hypercholesterolemic dogs by four methods: the fecal recovery method of Borgström (1969, *J. Lipid Res.* **10:** 331–337), the dual isotope method of Zilversmit and Hughes (1974, *J. Lipid Res.* **15:** 465–473), the recovery of cholesterol in thoracic duct lymph collected continuously for 16 hr after a meal, and the recovery of isotopic cholesterol from the liver and plasma 24 hr after the animals consumed an isotope-containing meal. The four methods showed excellent agreement and indicated that dogs fed a cholesterol-rich synthetic diet absorb 5.2 ± 0.5 g (mean ± SD) of cholesterol per day and that cholesterol absorption is reasonably constant from week to week in these animals. Separate estimates of cholesterol excretion indicated that these dogs excreted 4.7 ± 0.5 g of cholesterol per day, and thus were at or near the steady-state with regard to cholesterol input-output. These data, taken together with a previous report (1981, *J. Lipid Res.* **22:** 598–609), indicate that the canine liver can clear up to 300 mg of chylomicron cholesterol/hr, and support the concept that chylomicron remnants do not contribute significantly to the hypercholesterolemia in these animals. —**Melchior, G. W., and J. F Harwell.** Cholesterol absorption and turnover in hypercholesterolemic dogs. *J. Lipid Res.* 1985. **26:** 306–315.

Supplementary key words hypercholesterolemic dogs • β-sitosterol • chylomicrons • bile salt-lecithin-cholesterol micelles

Dogs are a widely used model for the study of hyperlipoproteinemia and its relationship to the deposition of lipid in the arteries (1–6). Dogs differ from some of the other models, e.g., the rabbit and nonhuman primate, in that they are very resistant to a diet-induced hyperlipoproteinemia. The two most widely used methods for producing hypercholesterolemia in dogs are feeding a synthetic diet (7) rich in cholesterol and with coconut oil as the only source of fat, or feeding a saturated fat-high cholesterol diet in combination with propylthiouracil (PTU) and bile salt (8). With either regimen, a severe hypercholesterolemia (ranging from 500 to greater than 1200 mg/dl) is usually produced, characterized by the appearance of β-VLDL, a marked increase in the levels of LDL, an increase in the apoE-containing HDL (HDL_c), and a decrease in the non-apoE-containing HDL (6). The apoprotein concentrations, as measured immunochemically, vary to some extent with the type of atherogenic regimen used but, in general, apoB levels increase 10- to 12-fold, the apoE levels 10- to 30-fold, and the apoA-IV levels 3- to 10-fold (9). The apoA-I levels decrease dramatically in the PTU-fed dogs to less than 40% of their normal levels within 3 weeks, but appear to increase in dogs fed the synthetic diet and remain elevated in those animals for at least 8 weeks (9).

Melchior, Mahley, and Buckhold (10) showed that chylomicron remnant clearance in hypercholesterolemic dogs was very similar to that in normal dogs and, thus, that cholesteryl ester-containing lipoproteins of intestinal origin made little direct contribution to the hypercholesterolemia in those animals. Hui, Innerarity, and Mahley (11) and Mahley et al. (12) showed that these animals have two types of hepatic lipoprotein receptors (apoE receptors and apoB, E receptors) and Angelin et al. (13) subsequently presented evidence that it was the hepatic apoE receptor that was primarily responsible for clearing these intestinal lipoproteins in the dog. Angelin et al. (13) also showed that the apoE receptors had a relatively large capacity for removal of chylomicron remnants (up to 250 mg of chylomicron cholesterol/hr); however, to date, no in vivo measurements have been made of the rate at which cholesterol in chylomicron remnants is presented to the hepatic receptors for removal. The present study was designed to quantify cholesterol absorption in these hypercholesterolemic dogs and, assuming that the newly absorbed cholesterol is rapidly and completely cleared from the plasma by the liver, to make preliminary estimates of the rate at which these hepatic receptors clear cholesterol from the circulation in intact animals.

[1]To whom reprint requests should be addressed. Present address: The Upjohn Company, 7250-209-4, Kalamazoo, MI 49001.

METHODS

Animals and diets

Adult, purebred foxhounds (four male and two female) were fed a previously described (7) high-cholesterol diet containing hydrogenated coconut oil as the sole source of fat (42% of calories) and cholesterol at a level of 15 mg/Cal. The animals had consumed their diets for 6–12 months, and their plasma cholesterol levels had plateaued before they were selected for experiments. A separate group of foxhounds (one male and one female) fed dog chow was used as controls. Diet consumption records were kept for each dog for the duration of the study.

Isotopes

[1,2-^{3}H]Cholesterol, [4-^{14}C]cholesterol, and [4-^{14}C]β-sitosterol were obtained from Amersham Searle Corporation (Arlington Heights, IL). The radiopurity of each isotope was checked by TLC on silica gel H plates developed in hexane–diethyl ether 50:50 and found to exceed 95%.

Absorption measurements

Cholesterol absorption was measured in four of the cholesterol-fed dogs (three male and one female) and the two control dogs by the fecal recovery method of Borgström (14) and the dual isotope method of Zilversmit and Hughes (15). Absorption was estimated in two separate cholesterol-fed dogs by collecting thoracic duct lymph for 16 hr after the high-cholesterol meal and measuring its cholesterol content.

Cholesterol absorption was estimated by the fecal recovery method by feeding the dogs 75 μCi of [1,2-^{3}H]cholesterol and 20 μCi of [4-^{14}C]β-sitosterol. The isotopes were dissolved in ethanol, added to about 3 oz of canned dog food, and fed to the dogs after they had consumed half of their assigned diet. The animals were then given the remainder of their assigned diet. This procedure assured us that all of the isotope would be consumed, and increased the probability that the isotope would mix completely with the cholesterol consumed at the particular meal.

Complete fecal collections were made daily thereafter for 7 days. The feces were collected in 1-liter lyophilizer jars, frozen, and lyophilized. The lyophilized feces were weighed and homogenized with a mortar and pestle, and duplicate 1-g aliquots were taken for analysis. The aliquots were added to 30 ml of 6 N NaOH in 33% ethanol and refluxed for 1 hr. The mixture was allowed to cool, 20 ml of water was added, and the mixture was extracted three times with 30 ml of hexane. The hexane extracts were pooled and brought to 100 ml final volume, and 4-ml aliquots were taken for radioactivity measurements. The hexane aliquots were added to scintillation vials and evaporated. Ethanol (0.5 ml) was added to each vial to resolubilize the lipids, after which 15 ml of Biofluor (New England Nuclear, Boston, MA) was added and the contents were mixed well. The radioactivity was measured as described below. The coefficient of variation, based on duplicate samples (N = 42 pairs) was 4.1% for cholesterol analysis and 4.5% for β-sitosterol analysis. The duplicates were not paired for analysis; rather, the samples were analyzed in random order in such a manner that, in some instances, duplicates were analyzed as much as 2 weeks apart.

The percent of the isotopic cholesterol absorbed was determined from the equation:

$$\% \text{ absorbed} = 1 - \frac{(1,2\text{-}^3\text{H})\text{cholesterol recovered}}{(4\text{-}^{14}\text{C})\beta\text{-sitosterol recovered}} \times \frac{(4\text{-}^{14}\text{C})\beta\text{-sitosterol consumed}}{(1,2\text{-}^3\text{H})\text{cholesterol consumed}} \quad \text{Eq. } 1)$$

The absolute mass of dietary cholesterol absorbed from the isotope-containing meal was determined from the equation:

$$\text{mass absorbed (g)} = \% \text{ absorbed} \times \text{cholesterol consumed (g)}. \quad \text{Eq. } 2)$$

Beta-sitosterol recovery was essentially complete within 48 hr after the isotope-containing meal (see Results); therefore, isotopic cholesterol recovered after that time was assumed to be cholesterol recycled from the bile, and was not included in the "[1,2-^{3}H]cholesterol recovered" component in Eq. *1*.

To estimate cholesterol absorption by the dual isotope method (15), we injected the dogs with 50 μCi of [4-^{14}C]cholesterol intravenously, and immediately thereafter fed them 300 μCi of [1,2-^{3}H]cholesterol, exactly as described for the fecal recovery method. The [4-^{14}C]cholesterol was solubilized in bile salt-lecithin-cholesterol micelles for injection. That solution was prepared by adding the isotope to a scintillation vial containing 54 mg of lecithin and 5 mg of cholesterol (Sigma Chemical Co., St. Louis, MO) dissolved in 1 ml of chloroform. After addition of the isotope, the chloroform was evaporated under N_2, 1 ml of 0.2 M sodium taurocholate solution (prepared by dissolving sodium taurocholate in phosphate buffered saline, pH 7.4) was then added, and the vial was swirled gently at 37°C until all of the lecithin and cholesterol were in solution (15–20 min). This synthetic bile solution was allowed to stand overnight at room temperature and then passed through a 0.22 μm filter (Millipore Corp., Bedford, MA). An aliquot was taken for radioactivity measurement, and the remainder was injected intravenously into the cephalic vein of the dog's forelimb.

The fraction of the dietary cholesterol absorbed from the isotope-containing meal was determined essentially as

described by Zilversmit and Hughes (15). Briefly, the plasma cholesterol specific activity of both isotopes was followed for 6–8 weeks, and the $\log_e$ plasma cholesterol specific activity was plotted versus time. The area under the resulting curves, between the limits t = 0 and t = 6, 7, or 8 weeks, was determined by planimetry using a HIPAD Digitizer (Houston Instrument, Austin, TX) connected to an Apple II computer (Apple Computer, Inc., Cupertino, CA). The fraction of the orally administered isotope absorbed was determined from the ratio of the area under the specific activity curve of the orally administered isotope to that of the intravenously administered isotope, after adjusting for differences in the amount of each isotope administered. This particular approach was necessary because the specific activity of the orally administered isotope often did not reach its maximum levels in the plasma until 48–96 hr after it was consumed by cholesterol-fed dogs, thereby indicating that the entry of the two isotopes into the various metabolic compartments was not in synchrony (see Results). The absolute mass of the cholesterol absorbed from that meal was determined just as for the fecal recovery method (Eq. *2*).

To estimate cholesterol recovery by the lymph recovery method, we fed [4-^{14}C]cholesterol (exactly as described for the fecal recovery method) to two foxhounds in which a lymph fistula had been created surgically, and collected the lymph for at least 16 hr. The procedure for creating the fistula and collecting the lymph has been described previously (10). Those analyses indicated that all of the newly absorbed cholesterol was contained in lymph lipoproteins of d < 1.006 g/ml (see Results); therefore, cholesterol absorption was assumed to be that recovered from the d < 1.006 g/ml fraction of lymph collected continuously for 16 hr.

Liver and bile analysis

The dogs were anesthetized with a mixture of nitrous oxide and halothane, a midline incision was made to expose the liver and gallbladder, and a small piece of liver (0.2–0.5 g) and 1 ml of bile were removed from each animal. Before analysis, the liver sections were blotted and weighed. The liver and bile samples were digested in 5 ml of 6 N KOH in 33% ethanol at 37°C for 24 hr. Three ml of water was added to each vial, and the neutral sterols were extracted with three 10-ml portions of hexane. The hexane extracts were pooled and duplicate aliquots were taken for cholesterol and radioactivity determinations. The radioactivity in the bile acid fraction of some samples was determined by titrating the solution remaining after neutral sterol extraction to neutral pH with HCl, measuring the final volume, and taking 0.5-ml aliquots for liquid scintillation counting.

Hepatic cholesterol and radioactivity concentrations were expressed per mg wet weight, and the total cholesterol and radioactivity content was taken as the product of their concentrations and the liver weight. In short-term experiments (after which the dog was killed), the liver weight was determined directly. In long-term experiments, which required that the dog remain alive for several weeks after the surgical biopsies were obtained, the liver weight was assumed to be 4.3 ± 0.4% of the dog's body weight, a value we established previously using six euthyroid, chronically hypercholesterolemic dogs fed the same diet as used in the present study.

Radioactivity determinations

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

Cholesterol determinations

The cholesterol content of the plasma and hexane extracts of the tissues was measured by the method of Abell et al. (16).

RESULTS

Cholesterol absorption was estimated in six hypercholesterolemic and two control dogs to determine to what extent daily cholesterol input was increased in euthyroid dogs consuming an atherogenic diet. **Table 1** shows the results of those absorption measurements in four of the hypercholesterolemic dogs and two control dogs. Cholesterol absorption was estimated in each of the dogs by two methods, the dual isotope method of Zilversmit and Hughes (15) and the fecal recovery method of Borgström

TABLE 1. Cholesterol absorption in hypercholesterolemic and control foxhounds

Dog Number	Plasma Cholesterol	Method			
		Fecal Recovery		Dual Isotope	
	mg/dl	%[a]	*g*[b]	%[a]	*g*[b]
Cholesterol-fed					
2168	643	20	6.0	17	5.1
2160	846	24	7.2	20	6.0
2814	904	36	5.5	33	5.4
1797	1245	13	3.9	15	4.5
Mean	921	23	5.7	21	5.3
Controls					
3108	131	88	0.2	70	0.2
5364	147	89	0.2	76	0.2
Mean	139	89	0.2	73	0.2

[a]Percent of the orally administered isotope absorbed.
[b]Mass of cholesterol absorbed from the isotope-containing meal.

(14). The two methods showed excellent agreement and indicated that dogs fed this diet absorb 4–6 g of cholesterol/day.

The fecal recovery method estimates cholesterol absorption from a single meal by feeding the animal isotopic cholesterol and subtracting the isotope recovered in the feces, during a given interval, from that ingested. The difference is assumed to be isotope that was absorbed by the animal. Corrections for bacterial degradation of cholesterol in the intestine and losses due to incomplete fecal collections are made by using β-sitosterol, a plant sterol very similar to cholesterol in structure but very poorly absorbed by most mammalian species (17). **Table 2** shows the mean β-sitosterol recovery, as well as its excretion pattern, for the animals used in this study. On the average, 95% of the recovered β-sitosterol had been recovered within 48 hr after the isotope-containing meal. In no instance was more than 6% recovered after 48 hr. As a result, we assumed that the isotopic cholesterol recovered by 48 hr, after being corrected for β-sitosterol recovery, was unabsorbed cholesterol.

The dual isotope method, as used here, estimates cholesterol absorption by feeding one isotope of cholesterol with the animals' meal while simultaneously administering the other intravenously and following the plasma specific activity of each isotope for several weeks. The fraction of the dietary cholesterol absorbed is determined from the ratio of the areas under the specific activity curves. In the present study, the isotope to be administered intravenously was dissolved in bile salt-lecithin-cholesterol micelles before injection. **Fig. 1** shows that the isotope administered in bile micelles is rapidly cleared from the plasma compartment, but a relatively small percentage appears in the liver (20–25%). Of that, 12% (which is equivalent to 2.2% of the radioactivity administered) was recovered in the bile of these animals within the first 6 hr after its injection. Thus, excessive amounts of the isotope were not lost in the bile before its equilibration with cholesterol in the rapidly miscible pool (18). **Table 3**

TABLE 2. The recovery of β-sitosterol in the feces after its consumption by dogs

Diet	N	Total Recovery[a]	Daily Recovery[b] 1	2	3	4	5	6
		%	%					
Atherogenic	4	73.6[c] (22.5)[d]	63.2 (23.2)	32.2 (22.2)	2.0 (1.2)	2.0 (0.7)	0.6 (0.4)	0.3 (0.3)
Control	2	73.5 (20.6)	85.5 (4.3)	13.0 (4.0)	0.8 (0.2)	0.2 (0)	0.5 (0)	0.1 (0.1)

[a]Percent of administered β-sitosterol recovered.
[b]Percent of the recovered β-sitosterol that was recovered on a given day.
[c]Mean.
[d]Half the range.

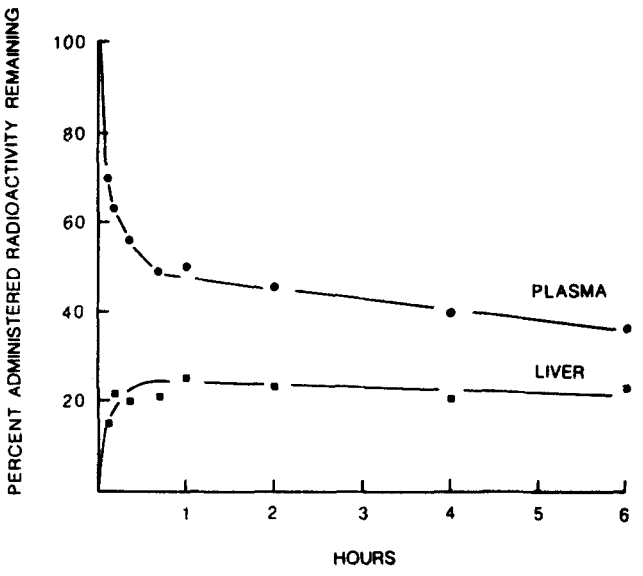

Fig. 1 Turnover of (4-^{14}C]cholesterol solubilized in taurocholate-lecithin-cholesterol micelles and administered intravenously to a hypercholesterolemic dog. Separate studies, using radiolabeled taurocholate, showed that 97% of the taurocholate was removed from the circulation within 15 min. In no instance was more than 3% of the administered cholesterol radioactivity recovered in the bile during the 6-hr interval.

shows that, within a few hours after administration of the bile micelles, the isotopic cholesterol distribution among the lipoproteins was very similar to that of the endogenous cholesterol, although a considerably smaller percentage was esterified. The free:ester ratio of the isotopic cholesterol, however, was, in every instance, identical to that of the endogenous cholesterol by 24 hr (data not shown). These data indicate that bile salt-lecithin-cholesterol micelles are a satisfactory vehicle for the intravenous administration of the isotope.

Fig. 2 shows specific activity dieaway curves typical of those obtained from control and hypercholesterolemic recipients fed [1,2-^{3}H]cholesterol and into which the [4-^{14}C]cholesterol-containing bile micelles had been injected. Note that in the hypercholesterolemic dog, the specific activity of the orally administered isotope did not reach its maximum value in the plasma until 48 hr after it was consumed, whereas in the control dog, the specific activity had peaked within 24 hr.

This delay in the hypercholesterolemic dogs was an absolutely consistent finding, ranging in duration from 48 to 96 hr in 12 chronically hypercholesterolemic dogs studied to date, and was the primary reason for comparing the ratio of the area under the curves, rather than random plasma samples (19), for estimating cholesterol absorption in those animals. The reason for this delayed appearance of the orally administered isotope is not clear, but it indicates that the dietary cholesterol is temporarily sequestered in some compartment distinct from, but in equilibrium with the plasma. Collection of thoracic duct lymph for 12 hr from two dogs with lymph fistulas showed

TABLE 3. Comparison of the [4-^{14}C]cholesterol distribution with the total cholesterol distribution among four plasma density fractions 4 hr after the intravenous administration of isotope-containing bile micelles

	Density (g/ml)			
	d<1.006	1.006-1.020	1.020-1.063	1.063-1.21
	%			
Radioactivity	12.3	14.3	43.8	29.2
	(6.4)[a]	(3.1)	(4.2)	(30.2)
Cholesterol	14.4	16.2	54.1	15.3
	(84.0)	(73.7)	(64.6)	(74.8)

[a]Percent esterified.

that essentially all of the newly absorbed cholesterol was contained in chylomicrons of d < 1.006 g/ml (Table 5, discussed below), and that recovery of the absorbed cholesterol was essentially complete by 16 hr (data not shown). This observation is in agreement with a previous report (10) showing that retinol and triglyceride absorption (both a measure of chylomicron production) were also complete within 12-16 hr after a meal. Thus, the intestine does not appear to be the extravascular compartment responsible for the delayed appearance of dietary cholesterol in the plasma. To confirm this, we bypassed absorption in one hypercholesterolemic dog by injecting intravenously 240 ml of thoracic duct lymph containing [4-^{14}C]cholesterol. The lymph, obtained from a hypercholesterolemic donor, was infused over a 2-hr period to the unanesthetized recipient. The change in plasma cholesterol specific activity over the ensuing 80 hr is shown in **Fig. 3.** The first plasma sample was taken 30 min after the infusion had stopped, and the plasma cholesterol specific activity continued to increase thereafter for the next 12 hr. The cholesterol specific activity appeared to peak between 12 and 18 hr after the infusion had stopped which, if one assumes absorption requires 8-12 hr (10), would correspond to roughly 26 hr after the meal. This observation supports the contention that the delayed appearance of dietary cholesterol in the plasma of chronically hypercholesterolemic dogs is not due to a slowed absorption in those animals.

Short-term studies using chronically hypercholesterolemic dogs suggested that chylomicron remnants were

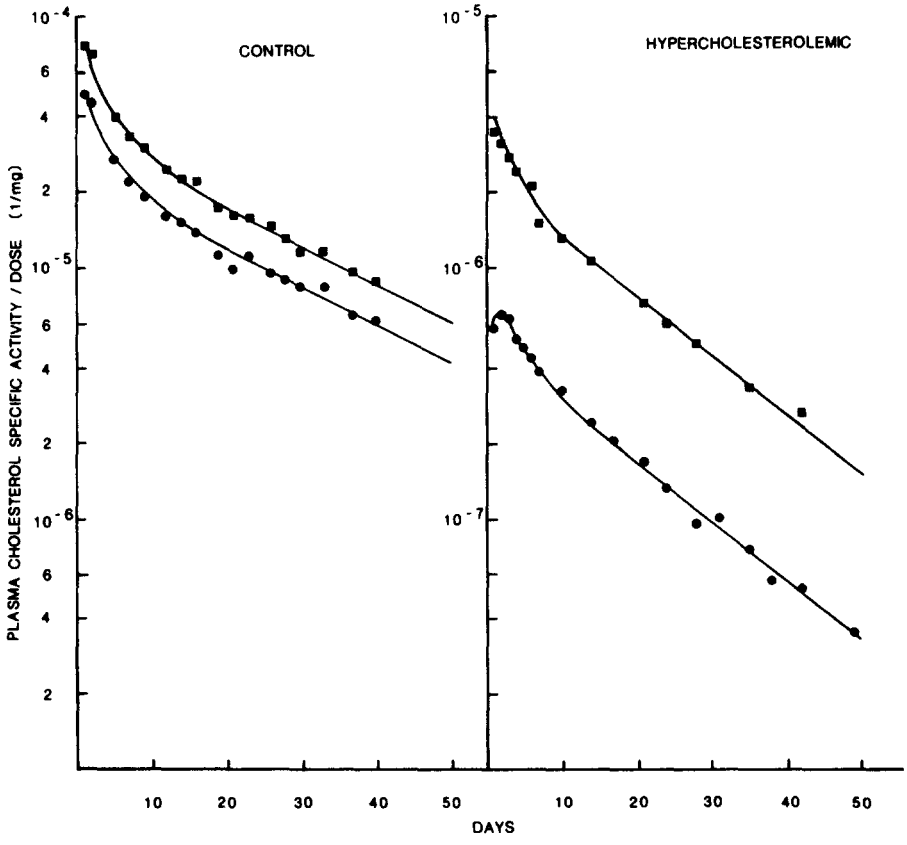

Fig. 2 Change in plasma-cholesterol specific activity in a normal and hypercholesterolemic dog. Two isotopes of cholesterol were administered simultaneously to each animal, one orally (●) and one intravenously (■) (see Methods). The specific activity was normalized by dividing it by the quantity of each isotope administered (note the difference in the scale of the ordinate between the normal and hypercholesterolemic dog). The fraction of the orally administered isotope absorbed was determined from the ratio of the areas under the curves between the limits t = 0 and t = 7 weeks.

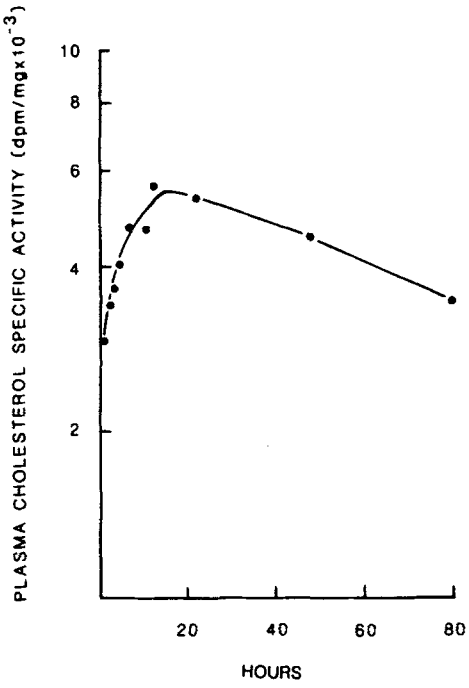

Fig. 3 Change in plasma-cholesterol specific activity after 240 ml of thoracic duct lymph, obtained from a donor fed [4-^{14}C]cholesterol, had been administered intravenously to a hypercholesterolemic recipient. The first plasma sample was taken 30 min after the lymph infusion had stopped. More than 85% of the administered radioactivity had been cleared from the plasma by that time.

rapidly cleared by the liver of those animals (10). To determine whether the liver might be the extravascular compartment responsible for the delayed appearance of the dietary cholesterol in the plasma of the hypercholesterolemic dogs, we compared the [4-^{14}C]cholesterol content of the liver and plasma in three dogs 24 hr after they had consumed a high-cholesterol meal containing the isotope. The results (**Table 4**) show that at 24 hr, about two-thirds of the newly absorbed cholesterol can be accounted for in the liver and plasma, with more than 75% in the liver. Note also that the hepatic cholesterol concentration (10.9 mg/g wet wt) in the chronically hypercholesterolemic dogs is considerably higher than the 2.6 mg/g wet weight reported previously for dogs (20).

Subsequent plasma and liver samples showed that the plasma cholesterol specific activity continued to climb as the hepatic cholesterol specific activity decayed and that the hepatic cholesterol specific activity intersected the plasma cholesterol specific activity curve near its apex, between 24 and 74 hr after the meal (**Fig. 4**). Thereafter, in every case, the respective specific activities were similar and appeared to decay in parallel. These data (Table 4 and Fig. 4) indicate that a markedly expanded hepatic cholesterol pool in the chronically hypercholesterolemic dogs is probably responsible for the delayed appearance of dietary cholesterol in the plasma.

Table 4 also contains an independent estimate of cholesterol absorption in these three dogs, obtained by dividing the μCi of [4-^{14}C]cholesterol recovered in the liver and plasma by the specific activity of the cholesterol consumed by the animal. This method probably underestimates the actual absorption to some extent, since a portion of the absorbed isotope was probably excreted within the first 24 hr after the meal; nonetheless, the estimate contained in Table 4 is very similar to that in Table 1 and indicates that our estimates of cholesterol absorption in these animals are, in fact, reasonable.

Finally, to confirm the values for cholesterol absorption shown in Tables 1 and 4, we measured the cholesterol recovered from thoracic duct lymph from two separate hypercholesterolemic dogs that was collected continuously for 16 hr after a meal (**Table 5**). Essentially all (98%) of the dietary cholesterol was contained in lipoproteins of $d < 1.006$ g/ml in the thoracic duct lymph, whereas only 80% of the total cholesterol was associated with lipoproteins of $d < 1.006$ g/ml. This finding indicates that 20% of the cholesterol in thoracic duct lymph of hypercholesterolemic dogs was probably not derived form that particular meal, i.e., it represents cholesterol in lymph draining from the periphery or cholesterol synthesized in the

TABLE 4. The recovery of [4-^{14}C]cholesterol from the liver and plasma 24 hr after it was consumed by chronically hypercholesterolemic dogs[a]

	Liver				Plasma				
N	Wet Wt.	Cholesterol Content	Dietary Cholesterol Recovered[b]		Volume	Cholesterol Content	Dietary Cholesterol Recovered[b]		Total Cholesterol Absorbed
	g	*g*	*μCi*	*g*	*ml*	*g*	*μCi*	*g*	*g*
3	1061[c]	11.6	12.8	3.8	1055	8.4	3.7	1.1	5.0
	(117)	(4.8)	(5.4)	(1.6)	(120)	(1.5)	(1.5)	(0.5)	(2.1)

[a]The liver biopsies and plasma samples were taken from dogs 2160, 2168, and 2814 (Table 1).
[b]The mass of newly absorbed cholesterol recovered in the liver and plasma was taken as the ratio of *radioactivity recovered in each compartment: the specific activity of cholesterol in the diet.* The dietary cholesterol specific activity was 7400 dpm/mg.
[c]Mean and (standard deviation).

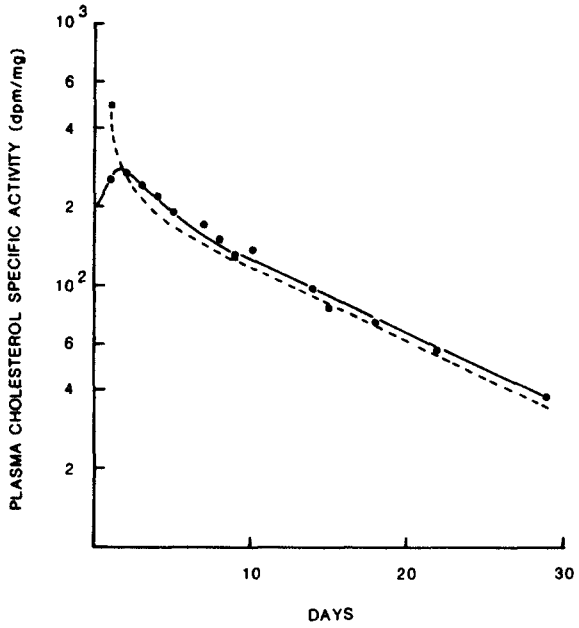

Fig. 4 Turnover of orally administered [4-^{14}C]cholesterol in the plasma (●) and liver (■) of a hypercholesterolemic dog. The dashed line represents the theoretical decay curve, assuming that the final exponential rate of decay of hepatic isotope is identical to that of the plasma.

intestine. Thus, we estimate that those animals absorbed 4.6 ± 1.5 g of dietary cholesterol within 16 hr after the meal, a value that agrees well with the 5.5-g estimate from Table 1 and the 5.0-g estimate from Table 3.

DISCUSSION

The purpose of this study was to quantify cholesterol absorption in hypercholesterolemic dogs consuming a widely used atherogenic diet. Cholesterol absorption was estimated using four independent methods, all of which agreed reasonably well with one another. The dogs consumed, on the average, 600 g of diet/day, which amounts to 30 g cholesterol/day. The average absorption was 17%, or 5.2 g/day. Although four independent methods of evaluating absorption were used in these studies, three of the methods (the fecal recovery method, the dual isotope method, and the recovery of isotope in the liver and plasma 24 hr after the meal) are based on the assumption that the orally administered isotope completely equilibrated with the dietary cholesterol before absorption. In the present study, the isotope was presented in a physical form (a few ounces of meat) different from the rest of the diet to assure that it was completely consumed by the animal. Thus, the possibility existed that the isotope was not equilibrating completely with the dietary cholesterol; however, to assure that our absorption estimates were, in fact, reasonable for this particular animal model, we also measured absorption by collecting thoracic duct lymph from two hypercholesterolemic dogs for an extended period after the high-cholesterol meal and obtained comparable results. Furthermore, the same four dogs were used to evaluate each method and those studies were done sequentially, 2–3 months apart. This fact not only indicates that the three methods give comparable, and thus probably correct results, but also suggests that cholesterol absorption is fairly stable from day to day in this model of hypercholesterolemia.

One of the problems that arise when considering an experiment requiring that cholesterol be administered intravenously is what vehicle one should use for the otherwise insoluble isotope. Two approaches currently used are injection of the isotope dispersed in ethanol-saline or injection as an emulsion stabilized with a detergent (21). In both instances, the isotope rapidly disappears from the plasma, presumably due to clearance by the reticuloendothelial system (21). Thus, certain assumptions must be made regarding the disposition of the phagocytized isotope, e.g., one must assume that all of the administered isotope taken up by the reticuloendothelial system is released back into the system under study; that all of it is released at about the same time (rather than spread out over several days); and that the released isotope equilibrates with the cholesterol in the system under study prior to its being excreted.

Preliminary studies suggested that the dog did not meet all of these criteria. Specifically, it appeared that sig-

TABLE 5. The distribution of total cholesterol and orally administered [4-^{14}C]cholesterol among five density fractions of thoracic duct lymph from two hypercholesterolemic dogs

		Density (g/ml)			
	Mass	d < 1.006	1.006–1.020	1.020–1.063	1.063–1.21
	g/day[a]		%		
Total cholesterol	5.5 ± 1.8[b]	82.5 ± 3.6	4.8 ± 0.4	4.7 ± 0.2	8.1 ± 2.0
[4-^{14}C]Cholesterol		98.3 ± 3.1	0.6 ± 0.1	0.4 ± 0.1	0.9 ± 0.1

[a]The total mass of cholesterol recovered from thoracic duct lymph collected continuously for 16 hr after a high cholesterol meal. Dietary [4-^{14}C]cholesterol absorption was greater than 90% complete by that time.
[b]Range/2.

nificant amounts of high specific activity cholesterol were suddenly appearing in the system as late as 2 weeks after isotope administration. This appeared as sharp increases in the plasma cholesterol specific activity during the period it should have been in the final exponential decay mode (data not shown).

We were also concerned that significant amounts of the isotope might be phagocytized by the Kupffer cells and subsequently picked up by the hepatic parenchymal cells and converted to bile salt or excreted without having a chance to equilibrate with the cholesterol in the rest of the system. This would reduce the size of the dose by an undetermined amount and thus conceivably create a significant error in the calculations.

As a result of these concerns, we chose to solubilize the cholesterol in a detergent micelle for injection. But, because commercially available detergents did not appear suitable, we decided to use bile micelles (vehicles whose components were all normal constituents of the body). Preliminary studies using radiolabeled taurocholate and cholesterol indicated that the micelle was disrupted within minutes after its injection (greater than 97% of the taurocholate was cleared within 15 min [data not shown]), whereas the turnover of the cholesterol was considerably slower. Excessive amounts of the isotope administered in bile micelles were not lost in the bile; there was no disappearance and subsequent reappearance of isotope in the plasma, as seen with particulate cholesterol; and the isotope in the plasma quickly equilibrated with the circulating lipoproteins and was esterified. Thus, this seemed a suitable alternative to the other methods of administering isotopic cholesterol intravenously.

One important observation from these studies was that the dual isotope method of Zilversmit and Hughes (15) does give accurate estimates of cholesterol absorption in the hypercholesterolemic dog. This method is by far the simplest and most straightforward of the various methods now in use to estimate cholesterol absorption. Nevertheless, because the appearance of the orally administered isotope in the plasma was consistently delayed in those animals, it was necessary to follow the plasma cholesterol specific activity of each isotope for several weeks and to compare the area under the respective specific activity versus time curves to obtain accurate results. A comparison of isotope ratios from random plasma samples resulted in overestimates of cholesterol absorption in all of the animals used in this study. Furthermore, the fact that the hepatic cholesterol specific activity was so much higher than that of the plasma at 24 hr (Table 4) indicates that, in this particular animal model, the plasma and liver are not only distinct anatomical compartments of cholesterol, but must also be considered distinct metabolic compartments when isotope kinetic data are evaluated.

Pertsemlidis, Kirchman, and Ahrens (22) quantified cholesterol absorption and synthesis in pedigreed dogs of comparable size to foxhounds that were fed a diet similar in many respects to that used here but which contained corn oil as the sole source of fat. Their dogs consumed 2.7–3 g of cholesterol/day and absorbed between 1 and 1.5 g cholesterol/day. That quantity of cholesterol, however, had relatively mild effects on the dogs' plasma cholesterol levels, raising the mean of the group from 184 mg/dl during the basal diet period to 290 mg/dl during the high-cholesterol diet period. Those investigators showed that dogs consuming their high-cholesterol diet compensated for the increased cholesterol input by an almost complete inhibition of cholesterol synthesis and by substantially increasing bile acid excretion.

We did not measure cholesterol synthesis directly in our study; however, at the end of the dual isotope experiments, we switched the diet of all four cholesterol-fed dogs to a fat-free, cholesterol-free chow for 7–10 days. During that period, the plasma specific activity stopped decaying in every animal and the slope of the curves became zero (data not shown). Although this does not prove absolutely that cholesterol synthesis was completely inhibited in our animals, it is the response one would expect if that were the case. Under that circumstance, absorption would be the sole source of cholesterol input in those dogs. Furthermore, if we assume that our animals were at or near a steady-state of cholesterol input-output, then we can assume that equivalent amounts of cholesterol were being excreted, i.e., that these animals were excreting 5.2 g of sterol (bile acid and neutral sterol) per day. We have two sources of evidence that our dogs were at or near the steady-state. First, their plasma cholesterol levels had stopped climbing and had remained reasonably steady for several months preceding the absorption measurements. Second, the animals used here for absorption measurements (Table 1) were injected intravenously with isotopic cholesterol on three separate occasions, the interval between injections being at least 2 months. When the plasma cholesterol specific activity dieaway curves were normalized by dose (as in Fig. 2), the respective curves from a given animal were virtually superimposable. Therefore, the size of the cholesterol pool diluting the isotope was not changing significantly during the study.

We also have independent evidence that the dogs were excreting about 5 g of sterols per day. That figure was derived from studies in which the [4-^{14}C]cholesterol was administered intravenously in bile micelles (Fig. 1). In both of the dogs used for those studies, 20–25% of the administered isotope was present in the liver within an hour after its administration and the hepatic cholesterol specific activity remained relatively constant for the ensuing 5 hr. Thus, the mass of hepatic cholesterol recovered in the bile during that interval could be estimated by dividing the total biliary radioactivity recovered by the mean hepatic cholesterol specific activity during that interval. We recovered an average of 2.2% of the admin-

istered radioactivity in the bile between 1 and 6 hr after its injection, and the normalized hepatic cholesterol specific activity (dpm/mg ÷ dpm administered) averaged 2.27×10^{-5}; thus, those dogs excreted an average of 969 mg of sterol in the bile every 5 hr, or 4.7 g sterol per day. This, of course, assumes that the isotope completely equilibrated with the hepatic cholesterol pool before excretion, an assumption that remains to be proved. Nonetheless, we believe it more than coincidental that these estimates agree so well with those obtained for absorption. Thus, even dogs absorbing these massive quantities of cholesterol are eventually able to compensate for the increased input by increasing their excretion, and a steady-state is apparently reestablished.

The question remains as to the mechanism by which the diet induces the hypercholesterolemia in these animals. This diet is one of the few that will produce hypercholesterolemia in euthyroid dogs. We have, in fact, fed dogs chow-base diets containing 5% by weight cholesterol and hydrogenated coconut oil as the sole source of fat and been consistently unable to produce a sustained hypercholesterolemia in those animals. Only after the animals were subjected to thyroidectomy did the hypercholesterolemia develop. Although cholesterol absorption was not measured in those animals, this observation suggests that the hypercholesterolemia was not due simply to their cholesterol excretion systems being overwhelmed by the increased influx of cholesterol, i.e., if we assume cholesterol absorption was the same before and after thyroidectomy, then it would indicate that the euthyroid animals were able to compensate for the marked increase in cholesterol absorption quickly and thereby maintain their plasma cholesterol concentration at or near basal levels, whereas the hypercholesterolemic animals were not and, thus, became hypercholesterolemic. We believe that a similar principle applies in the present situation. Even though the chow-based diet contained the same amount of cholesterol and the same fat as the synthetic diet, the animals did not become hypercholesterolemic. Thus, the synthetic diet used here must lack some necessary ingredient or contain some combination of ingredients that inhibits cholesterol clearance.

Melchior et al. (10) recently showed that the severe hypercholesterolemia that developed in foxhounds consuming this diet was not due to the accumulation of chylomicrons or chylomicron remnants in the plasma. In fact, the cholesteryl ester-rich chylomicron remnants appear to be rapidly cleared from the plasma by the liver, and control and chronically hypercholesterolemic dogs differ little in this respect. The data from the present study, showing that most of the newly absorbed cholesterol radioactivity was in the liver and not in the plasma 24 hr after the isotope-containing meals (Table 4), and that the plasma cholesterol specific activity did not peak until between 48 and 96 hr after consumption of the isotope by the hypercholesterolemic animals, appear to corroborate the original observation of Melchior et al. (10). It should be noted that this conclusion is not in conflict with a report by Fainaru et al. (23) indicating that chylomicron remnant cholesterol contributes to the β-VLDL cholesterol pool in hypercholesterolemic foxhounds. Although their data strongly suggest that chylomicron remnants are present, the contribution of these remnants to the hypercholesterolemia appears to be minor, accounting for 1–2% of the plasma cholesterol pool 12 hr after a meal (Ref. 23, Table 2). Furthermore, the plasma $t_{1/2}$ of the remnants appears to be very short (Ref. 23, Fig. 6), supporting the previous deduction of Melchior et al. (10) that the hepatic clearance mechanism of these remnants is extremely efficient, even in chronically hypercholesterolemic dogs.

If all the newly absorbed cholesterol enters the liver before being excreted or resecreted in lipoproteins, and if absorption is essentially complete within 16 hr, then our absorption data indicate that the canine liver can clear at least 300 mg of chylomicron cholesterol/hr from the plasma. This observation is in agreement with that of Angelin et al. (13), showing that their foxhounds could clear at least 250 mg of thoracic duct lymph cholesterol per hr. It is not clear whether this is the maximum rate at which chylomicron cholesterol can be cleared by the liver, since the rate-limiting step in this process may be the production of remnants in the periphery. Nonetheless, these data indicate that the canine liver has a large capacity for the clearance of chylomicron remnant cholesterol. Thus, the hypercholesterolemia that develops in these animals does not appear to be due to a diet-induced, hepatic lipoprotein receptor defect. Rather, the primary defect appears to reside within the hepatocyte. The rate-limiting step, at least initially, appears to be the capacity of the hepatocyte to excrete this cholesterol in the bile. As the levels of cholesterol in the hepatocyte increase, cholesterol presumably spills back into the plasma in lipoproteins of hepatic origin. It is conceivable that the clearance of those lipoproteins might be inhibited by down-regulated hepatic receptor levels (although that would clearly be a secondary defect), thus exacerbating the situation. To test that possibility, we switched chronically hypercholesterolemic dogs to a cholesterol-free, fat-free diet and found that their plasma cholesterol, apoB, and apoE levels decreased very rapidly (9), suggesting that no receptor defect existed or that the animals had compensated. Thus, when taken together with a previous report (10), this study suggests that the diet produces hypercholesterolemia in the dog by altering the sensitivity of the mechanism responsible for cholesterol excretion in the bile. Apparently, much higher levels of hepatic cholesterol are necessary to maximize cholesterol excretion in these dogs. Eventually, however, the animals are able to increase excretion substantially, since a steady-state is sub-

sequently re-established; but, at that point, the plasma cholesterol levels are extremely high.

This work was supported in part by the USPHS Program Project grant #HL08974. The authors gratefully acknowledge the excellent technical assistance of Ms. Jane Phung, Ms. Cindy Sood, and Mr. Joseph Taboada, and thank Ms. Wanda Cadwallader and Ms. Lawrie Lehmann for their help with the preparation of the manuscript and the Editorial Office, LSU Medical Center, for editing and processing the final copy.

Manuscript received 30 March 1984.

REFERENCES

1. Geer, J. C. 1965. Fine structure of canine experimental atherosclerosis. *Am. J. Pathol.* **47:** 241–269.
2. Mahley, R. W., A. W. Nelson, V. J. Ferrans, and D. L. Fry. 1976. Thrombosis in association with atherosclerosis induced by dietary perturbations in dogs. *Science.* **192:** 1139–1141.
3. 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.
4. 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. Pathol.* **87:** 205–226.
5. Sloop, C. H., L. Dory, R. Hamilton, B. R. Krause, and P. S. Roheim. 1983. Characterization of dog peripheral lymph lipoproteins: the presence of a disc-shaped "nascent" high density lipoprotein. *J. Lipid Res.* **24:** 1429–1440.
6. Mahley, R. W., and K. H. Weisgraber. 1984. Canine plasma lipoproteins: characterization of lipoproteins from control and cholesterol-fed dogs. *In* CRC Handbook of Electrophoresis, Vol. IV: Lipoproteins of Nonhuman Species. L. A. Lewis and H. K. Naito, editors. CRC Press, Boca Raton, FL. 151–173.
7. Malmros, H., and N. H. Stemby. 1968. Induction of atherosclerosis in dogs by a thiouracil-free semisynthetic diet containing cholesterol and hydrogenated coconut oil. *Prog. Biochem. Pharmacol.* **4:** 482–487.
8. Steiner, A., and F. E. Kendall. 1946. Atherosclerosis and arteriosclerosis in dogs following ingestion of cholesterol and thiouracil. *Arch. Pathol.* **42:** 433–444.
9. Melchior, G. W., L. Dory, and P. S. Roheim. 1984. Changes in plasma apo B, apo E, apo A-I, and apo A-IV concentrations in dogs consuming different atherogenic diets. *Atherosclerosis.* **52:** 47–57.
10. Melchior, G. W., R. W. Mahley, and D. K. Buckhold. 1981. Chylomicron metabolism during dietary-induced hypercholesterolemia in dogs. *J. Lipid Res.* **22:** 598–609.
11. Hui, D. Y., T. L. Innerarity, and R. W. Mahley. 1981. Lipoprotein binding to canine hepatic membranes. Metabolically distinct apo-E and apo-B,E receptors. *J. Biol. Chem.* **256:** 5646–5655.
12. Mahley, R. W., D. Y. Hui, T. L. Innerarity, and K. H. Weisgraber. 1981. Two independent lipoprotein receptors on hepatic membranes of dog, swine, and man. *J. Clin. Invest.* **68:** 1197–1206.
13. Angelin, B., C. A. Raviola, T. L. Innerarity, and R. W. Mahley. 1983. Regulation of hepatic lipoprotein receptors in the dog. Rapid regulation of apolipoprotein B,E receptors, but not of apolipoprotein E receptors by intestinal lipoproteins and bile acids. *J. Clin. Invest.* **71:** 816–831.
14. Borgström, B. 1969. Quantification of cholesterol absorption in man by fecal analysis after feeding of a single isotope-labeled meal. *J. Lipid Res.* **10:** 331–337.
15. Zilversmit, D. B., and L. B. Hughes. 1974. Validation of a dual-isotope plasma ratio method for measurement of cholesterol absorption in rats. *J. Lipid Res.* **15:** 465–473.
16. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1952. Simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* **195:** 357–366.
17. Grundy, S. M., E. H. Ahrens, Jr., and G. Salen. 1968. Dietary β-sitosterol as an internal standard to correct for cholesterol losses in sterol balance studies. *J. Lipid Res.* **9:** 374–387.
18. Goodman, D. S., and R. P. Noble. 1968. Turnover of plasma cholesterol in man. *J. Clin. Invest.* **47:** 231–241.
19. Zilversmit, D. B. 1972. A single blood sample dual isotope method for the measurement of cholesterol absorption in rats. *Proc. Soc. Exp. Biol. Med.* **140:** 862–865.
20. Pertsemlidis, D., E. H. Kirchman, and E. H. Ahrens, Jr. 1973. Regulation of cholesterol metabolism in the dog. II. Effects of complete bile diversion and of cholesterol feeding on pool sizes of tissue cholesterol measured at autopsy. *J. Clin. Invest.* **52:** 2368–2378.
21. Nilsson, Å., and D. B. Zilversmit. 1972. Fate of intravenously administered particulate and lipoprotein cholesterol in the rat. *J. Lipid Res.* **13:** 32–38.
22. Pertsemlidis, D., E. H. Kirchman, and E. H. Ahrens, Jr. 1973. Regulation of cholesterol metabolism in the dog. I. 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.
23. Fainaru, M., R. W. Mahley, R. L. Hamilton, and T. L. Innerarity. 1982. Structural and metabolic heterogeneity of β-very low density lipoproteins from cholesterol-fed dogs and from humans with Type III hyperlipoproteinemia. *J. Lipid Res.* **23:** 702–714.