

# Structural and metabolic heterogeneity of $\beta$ -very low density lipoproteins from cholesterol-fed dogs and from humans with Type III hyperlipoproteinemia

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**Abstract** Cholesteryl ester-rich  $\beta$ -very low density lipoproteins ( $\beta$ -VLDL) are  $\beta$ -migrating lipoproteins that accumulate in the  $d < 1.006$  g/ml fraction of plasma from cholesterol-fed animals and from patients with Type III hyperlipoproteinemia. They can be separated from pre- $\beta$ -migrating very low density lipoproteins in the  $d 1.006$  g/ml fraction by Geon-Pevikon block electrophoresis. The  $\beta$ -VLDL have a general property of stimulating cholesteryl ester synthesis and accumulation in macrophages. In the present study, we demonstrated that  $\beta$ -VLDL obtained from cholesterol-fed dogs fasted for 16 hr were heterogeneous and that two subpopulations of particles, referred to as Fractions I and II, could be isolated from the whole  $\beta$ -VLDL fraction using gel filtration chromatography. These fractions of  $\beta$ -VLDL were similar in that both were cholesteryl ester rich, had  $\beta$ -electrophoretic mobility on Geon-Pevikon electrophoresis, and possessed the B and E apoproteins as major constituents. However, Fractions I and II differed in size, shape, electrophoretic mobility, chemical composition, and apoprotein B type. (Fraction I vs. Fraction II: size: 90 to 300 nm vs. 20 to 70 nm; shape: irregular with redundant surface vs. spherical; electrophoretic mobility on paper: origin vs.  $\beta$ ; chemical composition: rich in phospholipid and poor in protein vs. rich in protein and poor in triglycerides; apoprotein B types: equal amounts of the high and low molecular weight forms vs. predominantly the high molecular weight form.) Furthermore, Fraction I was 3- to 15-fold more active than Fraction II in stimulating cholesteryl ester formation in mouse peritoneal macrophages. The concentration of Fraction I, but not Fraction II, was diminished in plasma by prolonged fasting, and Fraction I transported more intestinal-absorbed retinol than Fraction II. In addition, the plasma clearance of Fraction I injected into cholesterol-fed dogs was distinctly different from the clearance of Fraction II, and the in vivo dieaway of Fraction I resembled that of chylomicrons and chylomicron remnants. These findings suggest that  $\beta$ -VLDL in dogs are composed of cholesteryl ester-rich chylomicron remnants (Fraction I) and cholesteryl ester-rich lipoproteins, probably of liver origin (Fraction II). Finally, in studies of two patients with Type III hyperlipoproteinemia, we also identified the existence of two fractions in the  $\beta$ -VLDL with characteristics similar to Fractions I and II of cholesterol-fed dogs.—Fainaru, M., R. W. Mahley, R. L. Hamilton, and T. L. Innerarity. Structural and metabolic heterogeneity of  $\beta$ -very low density lipoproteins from cholesterol-fed dogs and from humans with Type III hyperlipoproteinemia. *J. Lipid Res.* 1982. 23: 702–714.

**Supplementary key words** dysbetalipoproteinemia • mouse peritoneal macrophages • cholesteryl esterification

The cholesterol feeding of animals, including man, causes marked changes in the plasma lipoproteins (1, 2). One of the experimental animal models used to investigate the pathogenesis of atherosclerosis is the cholesterol-fed dog (3–5). A characteristic accompaniment of diet-induced atherogenic hyperlipidemia, which is essential for the development of atherosclerosis in dogs, is the appearance of abnormal cholesterol-rich lipoproteins with the density of very low density lipoproteins ( $d < 1.006$  g/ml) (2, 5, 6). In contrast with normal triglyceride-carrying VLDL, which show pre- $\beta$ -mobility on electrophoresis and contain apoproteins B, E, and C, the cholesterol-rich VLDL, referred to as  $\beta$ -VLDL, have  $\beta$ -electrophoretic mobility and contain mainly apoproteins B and E (1). The  $\beta$ -VLDL from patients with Type III hyperlipoproteinemia (dysbetalipoproteinemia) shares these same characteristics (1, 7). The accumulation of  $\beta$ -VLDL in the plasma of cholesterol-fed animals is associated with the deposition of large amounts of cholesteryl esters in macrophages in a variety of tissues in vivo (6, 8), and these lipoproteins promote cholesteryl

Abbreviations: VLDL, very low density lipoproteins ( $d < 1.006$  g/ml) with pre- $\beta$ -mobility on electrophoresis;  $\beta$ -VLDL,  $d < 1.006$  g/ml lipoproteins with  $\beta$ -mobility on electrophoresis; LDL, low density lipoproteins containing only apoprotein B; HDL, high density lipoproteins; apo-E HDL<sub>c</sub>, cholesterol-induced HDL containing only apoprotein E; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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ester synthesis and accumulation in mouse macrophages and human monocytes in vitro (9, 10). The electrophoretic characteristics of  $\beta$ -VLDL have made it possible, using the method of Geon-Pevikon block electrophoresis (3, 4), to isolate  $\beta$ -VLDL from the pre- $\beta$ -VLDL, both of which occur in the  $d < 1.006$  g/ml fraction in plasma of cholesterol-fed animals.

To elucidate the origin and metabolic fate of  $\beta$ -VLDL, we have studied in detail the characteristics of these lipoproteins obtained from cholesterol-fed dogs and from patients with Type III hyperlipoproteinemia. The  $\beta$ -VLDL fraction in cholesterol-fed dogs and subjects with Type III hyperlipoproteinemia was found to be heterogeneous and composed of two distinct subpopulations of lipoproteins. These two subfractions, differing in size, shape, composition, and possibly origin, are described in the paper.

## MATERIALS AND METHODS

### Animals

Pure-bred male adult foxhounds (Brink Farm, Paola, KS), weighing 25 to 30 kg (20 to 30 months of age), were fed a semisynthetic diet prepared by Teklad Mills (Madison, WI). The diet consisted of 30% sucrose, 20% casein, 16% hydrogenated coconut oil, 5% cholesterol, 19.3% cellulose, 9% salt mixture, and 0.7% vitamin mixture (by weight) (5). The dogs were maintained on this diet, fed ad libitum, for a period of more than 1 year. Additional male foxhounds of similar breed, age, and weight were maintained on normal dog chow (Purina dog meal). Male and female Swiss-Webster mice (Simonson Lab., Inc., Gilroy, CA) weighing 25 to 30 g, were used as the source of peritoneal macrophages.

### Materials

Agarose A-15 m was purchased from Bio-Rad (Richmond, CA). New England Nuclear (Boston, MA) was the source of [ $1\text{-}^3\text{H}(\text{N})$ ]retinol (all *trans*, 5 Ci/mmol). Amersham/Searle was the source of [ $7(\text{n})^3\text{H}$ ]cholesterol (8 Ci/mmol) and [ $1\text{-}^{14}\text{C}$ ]oleic acid (56 mCi/mmol). Fetal calf serum was purchased from Sterile Systems, Inc. Dulbecco's phosphate-buffered saline (Cat. No. 450-1300), Dulbecco's modified Eagle's medium (DMEM) (Cat. No. 430-2100), potassium penicillin G, and streptomycin sulfate were purchased from GIBCO (Grand Island, NY). The plastic ware for tissue culture studies was obtained from Falcon (Becton, Dickinson, and Co.). Fucoidin was purchased from ICN (Cleveland, OH). All other supplies and reagents were obtained from sources as previously described (9-11).

## Lipoproteins

The dogs were fasted overnight (15 to 18 hr), and blood was drawn from the jugular vein into chilled tubes containing disodium EDTA (0.01% w/v final concentration, pH 7.4). All procedures involving lipoprotein isolation and characterization were started immediately after blood drawing and carried out at 4°C. Preparative ultracentrifugation was performed in a L8-70 Beckman ultracentrifuge (Beckman Instruments, Mountain View, CA). The  $d < 1.006$  g/ml fraction from cholesterol-fed dogs and VLDL of normolipidemic dogs were isolated at plasma density and washed in 0.15 M NaCl at  $d 1.006$  g/ml at 50,000 rpm in a 60 Ti rotor. LDL (1.02 to 1.063 g/ml) and HDL (1.125 to 1.21 g/ml) of normal dogs were isolated as described previously (10). LDL (1.02 to 1.063 g/ml) and apo-E HDL<sub>c</sub> (1.006 to 1.02 g/ml) were isolated from cholesterol-fed dogs by a combination of ultracentrifugation and Geon-Pevikon block electrophoresis, as described previously (4, 5). Dog chylomicrons ( $S_f > 400$ ) were obtained from thoracic duct lymph of normal dogs, as previously described (12).

The washed  $d < 1.006$  g/ml fraction ( $\sim 15$  mg of lipoprotein protein) isolated from cholesterol-fed dogs was subjected to Geon-Pevikon block electrophoresis (3, 4). The location of the pre- $\beta$  and  $\beta$  bands was visualized using an ultraviolet light, and the bands were removed from the block and eluted from the support medium with saline (4, 5). The pre- $\beta$ -migrating VLDL and the  $\beta$ -migrating  $\beta$ -VLDL obtained by these procedures were concentrated (PM-30 membrane in ultrafiltration cells (Amicon Corp., Lexington, MA)) and used for further characterization.

### Chromatographic separation of $\beta$ -VLDL

The  $\beta$ -VLDL (3 to 6 mg of lipoprotein protein in 3 ml of saline) were gently mixed with sucrose (20% w/v final concentration) and applied to a 4% (A-15 m) agarose column (2.2  $\times$  90 cm) equilibrated with 0.15 M NaCl, 10 mM sodium phosphate (pH 7.4) containing 0.01% (w/v) sodium azide. The column was equilibrated and operated at 4°C. Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used to determine the void volume, and lipoproteins obtained from both normal and cholesterol-fed dogs were used for further calibration of the column. The  $\beta$ -VLDL subfractions were eluted from the column in the column buffer at a constant flow rate (15 ml/hr) using a peristaltic pump (Varioperpex LKB). Fractions of constant volumes (3.75 ml) were collected at 15-min intervals. The fractions were extensively dialyzed against saline, 0.01% EDTA, pH 7.4, prior to use.

## Lipoprotein characterization

Paper electrophoresis was performed as previously described (4). Negative staining electron microscopy was performed using 2% potassium phosphotungstate (pH 6.4 to 6.5) (13). Electron micrographs were taken of random areas of several grids at a magnification of 20,000 using a Siemens 101 electron microscope (Siemens Corp., Medical/Industrial Groups, Iselin, NJ) (13). The diameters of 300 particles were measured from the photomicrographs enlarged at 60,000 diameters.

## Chemical determinations

Protein was determined by the method of Lowry et al. (14), using bovine albumin as the standard. Total cholesterol and triglyceride were determined using enzymatic procedures (Bio-Dynamics, Boehringer-Mannheim Corp.), and phospholipid content was determined from the phosphorus content (15). Cholesteryl esters were quantified by a combination of thin-layer chromatography and gas-liquid chromatography (Hewlett-Packard Model 5880). Lipoproteins were delipidated and electrophoresed on 4% or 11% polyacrylamide gels using sodium dodecyl sulfate as previously described (5, 16). The gels were stained with Coomassie blue.

## Incorporation of retinol into the plasma lipoproteins

Cholesterol-fed dogs were given [ $^3\text{H}$ ]retinol (300  $\mu\text{Ci}$ ) orally either by introduction of a gelatin capsule into the esophagus of the dog at feeding time or by mixing the [ $^3\text{H}$ ]retinol with the cholesterol-rich diet. In the latter case, cream was added to the coconut oil-cholesterol diet to ensure rapid consumption of the meal by the dog within a few minutes. In two of the cholesterol-fed dogs that received the retinol via capsule, approximately 15% of the administered dose was in the plasma at 18 hr.

## Cultured mouse macrophages

Mouse peritoneal macrophages were harvested from unstimulated mice using phosphate-buffered saline as described (9, 10). The peritoneal macrophages ( $1.5$  to  $3 \times 10^6$  cells per mouse) were pooled, and then were pelleted by centrifugation (400  $g$ , 10 min at room temperature). The cells were resuspended in DMEM containing penicillin (100 units/ml), streptomycin (100  $\mu\text{g}$ /ml), and 20% heat-inactivated fetal calf serum at a final concentration of  $1.5 \times 10^6$  cells per ml. The macrophages were dispensed into 16-mm plastic Petri dishes ( $8 \times 10^5$  cells per dish). After incubation in a humidified  $\text{CO}_2$  (7.5%) incubator for 2 hr, the dishes were washed three times with DMEM without serum to remove nonadherent cells. The cultured macrophages were incubated

18 to 24 hr at  $37^\circ\text{C}$  in 0.5 ml of DMEM containing 20% fetal calf serum. The cells were then washed once with DMEM and used for the determination of cholesteryl [ $1\text{-}^{14}\text{C}$ ]oleate synthesis by the procedure previously described (9, 10). At the end of each experiment (see figure or table captions for details of each experiment), the Petri dishes of macrophages were chilled on ice and then washed three times in rapid succession with cold phosphate-buffered saline (PBS), twice with PBS containing bovine serum albumin (2 mg/ml) for 10 min each, and then one short wash with cold PBS. The washed macrophages were extracted in situ with hexane-isopropanol 3:2 (v/v) for 30 min at room temperature (17). After the lipids had been extracted, the macrophages were dissolved with 0.1 N NaOH, and aliquots were removed for protein determination. The lipid extracts, blown dry with a stream of nitrogen, were resolubilized in chloroform-methanol 2:1 (v/v), spotted on Whatman LK6DF channelled preabsorbent TLC plates, and developed in hexane-diethyl ether-ammonium hydroxide 90:10:1 (v/v). The cholesteryl ester band, visualized with iodine vapor, was scraped into scintillation vials and counted using a dual-label counting program with the Beckman LS-9000.

## Lipoprotein modification and iodination

Human LDL,  $\beta$ -VLDL, and its fractions were labeled (200 to 400 cpm/ng) with either  $^{125}\text{I}$  or  $^{131}\text{I}$  by the iodine monochloride method (18). The free iodine was removed from radiolabeled lipoproteins by dialysis in 0.15 M NaCl containing 0.01% disodium EDTA. The  $^{125}\text{I}$ -labeled human LDL were acetylated exactly as described by Goldstein et al. (19).

## Plasma clearance of $\beta$ -VLDL fractions in cholesterol-fed dogs

Iodinated lipoproteins (0.1–0.5 mg of protein) were injected in the cephalic vein of conscious cholesterol-fed foxhounds. Blood samples were obtained from the jugular veins at the designated times and put into tubes containing disodium EDTA (0.01% w/v), and the plasma was separated promptly (3000 rpm  $\times$  20 min, at  $4^\circ\text{C}$ ). Aliquots of plasma (0.5–1.0 ml) were counted. Trichloroacetic acid (TCA)-precipitable activity in the plasma samples was determined by adding an equal volume of 20% TCA to plasma samples. The samples were vortexed, kept in ice for 20 min, and then centrifuged (3000 rpm  $\times$  20 min,  $4^\circ\text{C}$ ). An aliquot of supernatant was counted to determine nonprotein label. Calculations were based on plasma volume of 4.5% of body weight (12).

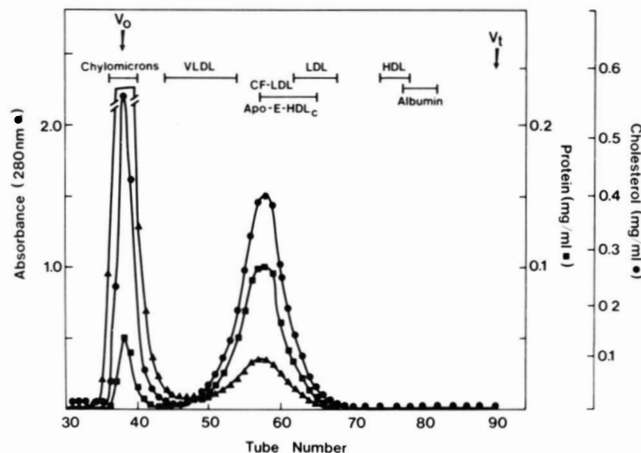


## RESULTS

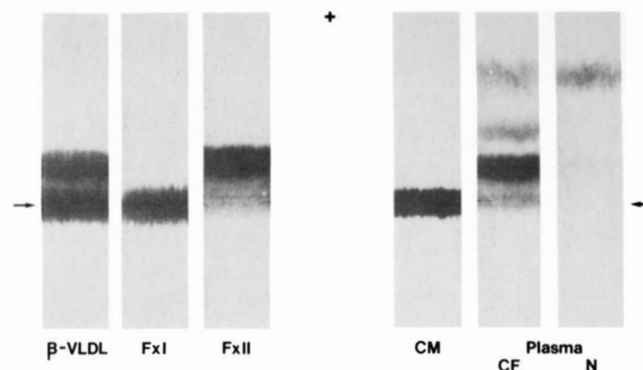
### Isolation of fractions from dog $\beta$ -VLDL

The  $d < 1.006$  g/ml fraction was obtained from the plasma of the cholesterol-fed dogs that were fasted 15–18 hr. These dogs had plasma cholesterol levels of 675 to 1000 mg/dl and triglyceride levels of 15 to 260 mg/dl. This  $d < 1.006$  g/ml fraction was subjected to Geon-Pevikon block electrophoresis for 18 hr at 4°C. Of the applied lipoprotein protein, 50–60% was recovered in two bands with pre- $\beta$  and  $\beta$ -mobility (5.5–7.5 and 7.5–9.0 cm from the origin, respectively). Approximately 85% of the recovered protein was associated with the band with  $\beta$ -mobility ( $\beta$ -VLDL) and the remainder with the pre- $\beta$ -migrating band (VLDL).

The  $\beta$ -VLDL obtained by Geon-Pevikon block electrophoresis were then subjected to gel filtration chromatography. More than 90% of the lipoproteins applied to the agarose column was recovered in two fractions (Fig. 1). The first fraction (Fraction I) eluted in the void volume, and the second fraction (Fraction II) eluted between normal VLDL and the high molecular weight LDL of the cholesterol-fed dogs. The two fractions (Fraction I, tubes 36 to 42; Fraction II, tubes 51 to 64, Fig. 1) were concentrated and used within 1 week for the various studies. Fraction I and Fraction II were pres-



**Fig. 1.** Gel filtration permeation chromatography of  $\beta$ -VLDL from cholesterol-fed dog. The  $\beta$ -VLDL were isolated from the plasma of fasting dogs by ultracentrifugation at  $d < 1.006$  g/ml and purified by Geon-Pevikon block electrophoresis. The  $\beta$ -VLDL were then applied to the agarose A-15 m column (4 mg of lipoprotein protein). The column was operated at 4°C with a constant flow of 16 ml/hr, and fractions of 3.75 ml were collected. Absorbance at 280 nm ( $\Delta$ — $\Delta$ ) and protein ( $\blacksquare$ — $\blacksquare$ ) and cholesterol ( $\bullet$ — $\bullet$ ) concentrations were determined on individual fractions. Blue Dextran 2000 was used for determination of void volume ( $V_0$ ). Other markers used for calibration were lymph chylomicrons, VLDL, LDL, and HDL isolated from normal dogs and LDL and apo-E HDL<sub>c</sub> of cholesterol-fed dogs.



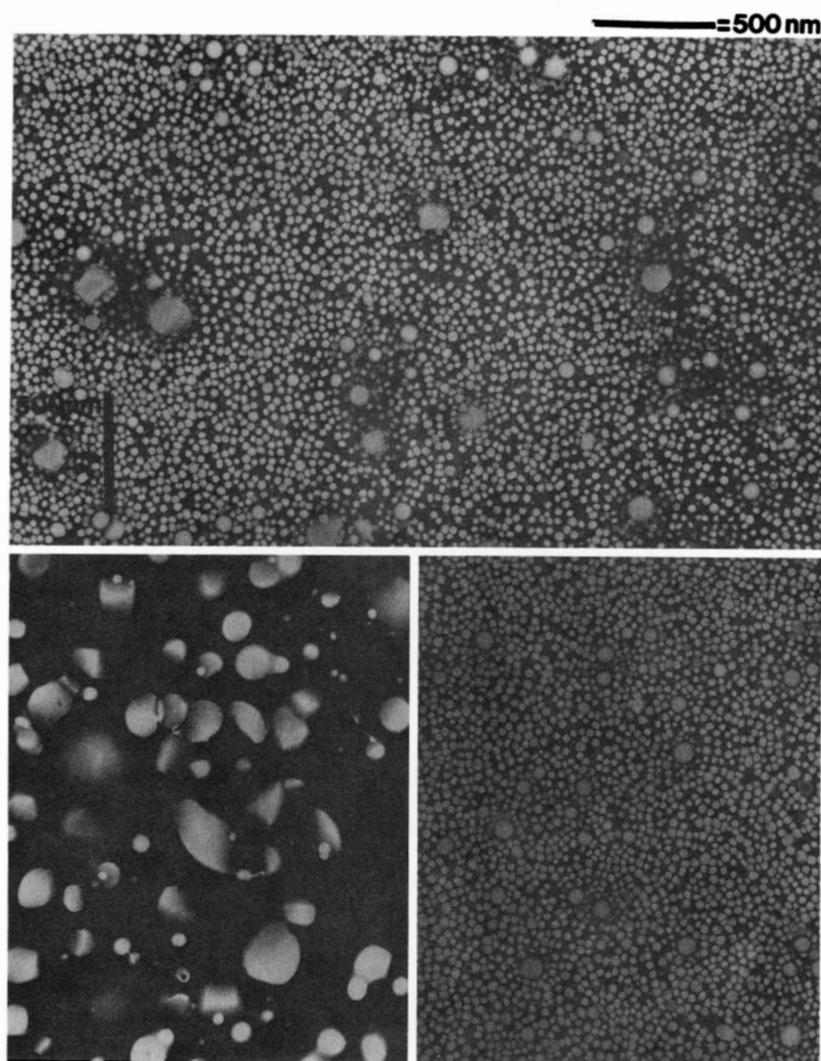
**Fig. 2.** Paper electrophoretograms of dog  $\beta$ -VLDL and the two fractions separated from  $\beta$ -VLDL on agarose A-15 m (Fig. 1). The fractions (Fx) were concentrated by ultrafiltration and 5–10  $\mu$ g protein were applied. For comparison, electrophoretograms of dog lymph chylomicrons (CM) and whole plasma from dogs (CF, cholesterol-fed; N, normal) are shown on the right.

ent in the  $\beta$ -VLDL fraction in approximately equal concentrations based on cholesterol content (51 vs. 49%); however, Fraction I accounted for 20% of the protein of the  $\beta$ -VLDL, whereas Fraction II accounted for 80% of the total  $\beta$ -VLDL protein.

### Characterization of $\beta$ -VLDL fractions in the dog

**Electrophoretic mobility.** With certain samples, the  $\beta$ -VLDL obtained from the Geon-Pevikon block could be resolved into two bands by paper electrophoresis (Fig. 2). One remained at the application zone similar to the electrophoretic behavior of dog chylomicrons isolated from thoracic duct lymph, and the other migrated with  $\beta$ -mobility similar to dog LDL. The two  $\beta$ -VLDL fractions obtained by gel filtration were resolved into these two bands (Fig. 2). Fraction I remained at the origin but the majority of Fraction II had  $\beta$ -electrophoretic mobility.

**Morphology and particle size.** Electron microscopic examination of the  $\beta$ -VLDL and its two chromatographically separable fractions revealed a marked morphologic heterogeneity (Fig. 3). The  $\beta$ -VLDL were a mixture of particle sizes (Fig. 3, top). Fraction I (bottom left) consisted of large particles (90–300 nm in diameter) with an irregular shape and characterized by the appearance of redundant surface material. This resembled the morphological characteristics of lymph chylomicrons and remnants (20). Some of the irregular forms observed in this fraction may have resulted from the crystallization of the triglycerides. This has been reported to occur when triglyceride-rich lipoproteins are cooled below 17°C (21). Approximately 90% of the particles in Fraction I ranged in size from 100 to 250 nm (mean  $\pm$  SD, 159.7  $\pm$  46.6; median 150.0 nm in diameter). By contrast, Fraction II



**Fig. 3.** Electron micrographs of negatively stained  $\beta$ -VLDL (top) obtained from a cholesterol-fed dog and fractions separated by gel filtration (see legend to Fig. 1). Fraction I (lower left) and Fraction II (lower right) are shown. The inserted bar represents 500 nm.  $\times 33,000$ .

was more homogeneous (Fig. 3, bottom right) and consisted of smaller spherical particles (20–75 nm). Approximately 85% of the particles in this fraction ranged in size from 20 to 50 nm (mean  $\pm$  SD,  $35.0 \pm 5.0$ , median 33.3 nm). The remaining particles were 60–75 nm in diameter (mean  $\pm$  SD,  $71.5 \pm 7.7$ ; 70.0 nm).

**Chemical composition and molecular weight.** Both Fractions I and II were cholesterol rich (44 vs. 59% by weight, respectively) but differed markedly in other constituents (Table 1). Fraction I lipoproteins contained more triglyceride and less protein than the Fraction II lipoproteins. The whole  $\beta$ -VLDL had an intermediate chemical composition, as expected. Based on the chemical composition (Table 1) and average molecular size, the average molecular weights for the two fractions were calculated according to Shen, Scanu, and Kezdy (22).

The calculated molecular weights for Fractions I and II were approximately  $340 \times 10^6$  and  $26 \times 10^6$ , respectively.

**Apoprotein composition.** The dog  $\beta$ -VLDL and the two fractions isolated by agarose chromatography had a similar apoprotein content when observed on 11% polyacrylamide gels. The major, and usually the only detectable apoproteins, were the B and E apoproteins (Fig. 4, left). However, differences in the apoprotein B were noted when the proteins were analyzed on 4% acrylamide gels (Fig. 4, right). The dog apo-B revealed a heterogeneity similar to that reported for apo-B of humans (23) and rats (24, 25). Dog lymph chylomicrons (CM) contained primarily, or exclusively, the low molecular weight apo-B form (L), whereas normal dog plasma LDL contained the higher molecular weight form (H,



TABLE 1. Chemical composition of dog  $\beta$ -VLDL and its fractions separated by chromatography

	Protein	Cholesterol	Triglyceride	Phospholipid
	% by weight			
Whole $\beta$ -VLDL <sup>a</sup>	10.3 $\pm$ 1.5	55.3 $\pm$ 5.9 (66.0) <sup>b</sup>	15.7 $\pm$ 3.1	18.7 $\pm$ 5.2
Fraction I <sup>a</sup>	3.3 $\pm$ 1.3	43.9 $\pm$ 10.3 (77.0) <sup>b</sup>	20.9 $\pm$ 9.7	31.9 $\pm$ 13.9
Fraction II <sup>a</sup>	14.5 $\pm$ 2.0	58.7 $\pm$ 6.9 (58.0) <sup>b</sup>	13.6 $\pm$ 3.4	13.1 $\pm$ 3.9

<sup>a</sup> The  $\beta$ -VLDL were isolated from the plasma of cholesterol-fed dogs fasted for 16 hr, as described in Materials and Methods. The ultracentrifugally washed  $d < 1.006$  g/ml fraction of plasma was subjected to Geon-Pevikon block electrophoresis and the  $\beta$ -VLDL was then subfractionated by gel filtration on agarose A-15 m into Fractions I and II. The results are expressed as percent composition (w/w) for five different preparations, each from an individual dog. The plasma from three different cholesterol-fed dogs was used during the course of the studies.

<sup>b</sup> Cholesteryl ester was determined on two occasions and the number in parentheses is the percent of the total cholesterol represented by the cholesteryl ester (the average of the two values).

Fig. 4). Fraction II of the  $\beta$ -VLDL contained predominantly the high molecular weight form of apo-B (H) and only very small amounts of the lower molecular weight form (L). By contrast, Fraction I contained approximately equal amounts of the high and low molecular weight forms of the apo-B as estimated visually from stained gels.

#### Effect of fasting on the dog $\beta$ -VLDL fractions

To determine the relationship between feeding and the concentration of the two  $\beta$ -VLDL subfractions, we subjected three dogs to prolonged fasting (24–48 hr). Fasting caused a drop in the cholesterol concentration in the plasma and in the  $d < 1.006$  g/ml fraction. Fraction I concentration was markedly reduced (greater than a 75% reduction), whereas the concentration of Fraction II decreased only slightly, if at all, with fasting for up to 48 hr (Table 2).

#### Transport of retinol in lipoproteins of cholesterol-fed dogs

To test further the possible intestinal origin of Fraction I, a dog maintained on the high cholesterol diet for approximately 6 months was fed a large meal of the coconut oil-cholesterol diet containing [<sup>3</sup>H]retinol (300  $\mu$ Ci), and the incorporation of the [<sup>3</sup>H]retinol into the  $\beta$ -VLDL fraction was determined. Cream was added to the meal to stimulate its immediate consumption by the dog. More than 96% of the [<sup>3</sup>H]retinol in the plasma, 5 hr after consumption of the meal, was present in the  $d < 1.006$  g/ml fraction. The agarose column profile showing the distribution of the [<sup>3</sup>H]retinol and total cholesterol within the fractions reveals the marked predominance of retinol with the Fraction I lipoproteins (Fig. 5).

In previous studies (12), we have observed that [<sup>3</sup>H]retinol was absorbed in cholesterol-fed dogs over a

period of many hours. In a separate study, two chronically cholesterol-fed dogs were given 300  $\mu$ Ci of [<sup>3</sup>H]retinol by capsule with one of their coconut oil-cholesterol meals (no cream), and the distribution of [<sup>3</sup>H]retinol was determined after 18 hr. At this time interval, more than 70% of the [<sup>3</sup>H]retinol was associated with the  $d > 1.006$  g/ml fraction. However, among the lipoproteins of the  $d < 1.006$  g/ml fraction, threefold more [<sup>3</sup>H]retinol was associated with Fraction I as compared with Fraction II lipoproteins (~20% of the retinol was in Fraction I and ~7% of the retinol was associated with Fraction II). Previously, we have suggested that

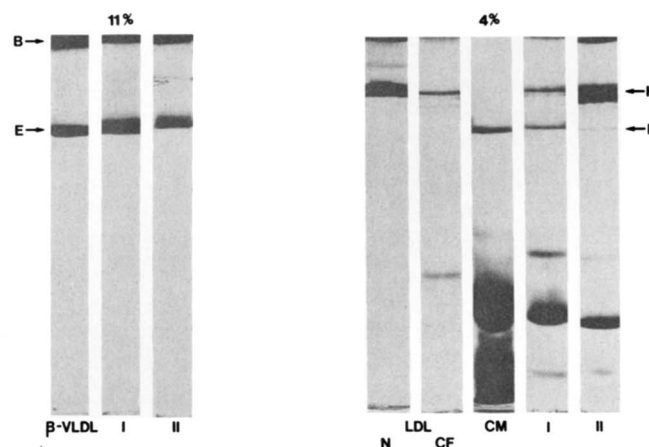


Fig. 4. SDS-polyacrylamide gel electrophoresis of dog  $\beta$ -VLDL and its fractions. On the left are three 11% gels. Apoproteins B and E were identified by comparison with canine apoproteins previously isolated and characterized (5). Equal amounts (30  $\mu$ g of lipoprotein protein) of  $\beta$ -VLDL and Fractions I and II were applied to the gels. On the right are 4% acrylamide gels. From right to left, Fractions II (II) and I (I) (40  $\mu$ g protein each) of dog  $\beta$ -VLDL are compared with dog lymph chylomicrons (CM) (80  $\mu$ g) and LDL (20  $\mu$ g) obtained from cholesterol-fed (CF) and normal (N) dogs. The high (H) and low (L) molecular weight bands coelectrophoresed with B-100 in human LDL and B-48 in human chylomicrons (23), respectively (not shown).

TABLE 2. Effect of fasting on plasma cholesterol and the fractions of  $\beta$ -VLDL from a cholesterol-fed dog<sup>a</sup>

Duration of Fasting	Cholesterol (mg/dl)			
	Plasma	d < 1.006 g/ml <sup>b</sup>	Fraction I <sup>b</sup>	Fraction II <sup>b</sup> +VLDL
12 h	692	22.9	11.4	11.0
24 h	684	20.4	8.1	11.9
48 h	548	12.5	1.7	10.3

<sup>a</sup> A cholesterol-fed dog was fasted for 48 hr (with free access to water) and 30-ml blood samples were withdrawn at the stated times. Plasma was separated immediately and centrifuged at d 1.006 g/ml (Materials and Methods). In this study, because of the relatively small amount of plasma obtained, the washed d < 1.006 g/ml fraction was applied directly to the gel filtration on agarose A-15 m. Cholesterol was determined on individual tubes and then the peaks were combined (as in Fig. 1). The VLDL represent a minor component of the d < 1.006 g/ml fraction (<15%) and were included in Fraction II (Fig. 1). Results are from a representative study.

<sup>b</sup> Cholesterol content is expressed as concentration (mg/ml) in the original plasma.

retinol can be incorporated into hepatic lipoproteins (e.g., VLDL) or into products of hepatic lipoproteins (e.g., LDL) following the recirculation of retinol from the intestine to the liver (12). This may account for the appearance of [<sup>3</sup>H]retinol in the Fraction II lipoproteins at this later time interval.

#### In vivo plasma clearance of the $\beta$ -VLDL fractions in cholesterol-fed dogs

Previously, we demonstrated that chylomicrons and chylomicron remnants were rapidly and efficiently cleared from the plasma of cholesterol-fed dogs (more than 90% of the injected dose was removed from the plasma in 1 hr) (12). The similarities between the physical and chemical properties of the Fraction I lipoproteins and those of chylomicrons and chylomicron remnants prompted us to determine the metabolic behavior of Fraction I injected into cholesterol-fed dogs. As shown in Fig. 6, the Fraction I lipoproteins were cleared from the plasma with kinetics similar to those previously described for the plasma clearance of chylomicrons (12). Furthermore, the unique differences between the Fraction I and Fraction II lipoproteins were further highlighted by the apparent divergence in mechanisms responsible for their clearance from the plasma. The plasma clearance of Fraction II lipoproteins was characterized by a sharp initial dieaway followed by a slower disappearance of the labeled lipoproteins from the plasma (Fig. 6). Essentially identical results have been observed using six different preparations of Fraction I and Fraction II lipoproteins injected into six cholesterol-fed dogs. Studies designed to characterize the mechanisms involved in the plasma clearance of these two subfractions of lipoproteins are in progress.

#### Characterization of lipoprotein subpopulations in d < 1.006 fraction of patients with Type III hyperlipoproteinemia

Two Type III hyperlipoproteinemic patients have been extensively studied. One patient (S.B.), a 45-year-old female homozygous for the E-2 apo-E (26) and phenotypic characteristics of Type III hyperlipoproteinemia, had not been on medication for several months and had d < 1.006 g/ml cholesterol and triglyceride concentrations of 142 and 262 mg/dl, respectively, at the time of the study. A second patient (D.R.), a 50-year-old male homozygous for the E-2 apo-E and with typical Type III hyperlipoproteinemia, was medicated with nicotinic acid and cholestyramine at the time of the study. Blood was obtained after a 14-hr fast and was ultracentrifuged at d < 1.006 g/ml, as described for preparation of the dog d < 1.006 g/ml lipoproteins. The d < 1.006 g/ml fraction, subjected to Geon-Pevikon block electrophoresis, was resolved into two bands, one with  $\beta$ -mobility ( $\beta$ -VLDL) and the other with pre- $\beta$  (VLDL) mobility. The  $\beta$ -VLDL and VLDL bands each represented approximately 50% of the recovered lipoprotein protein in the d < 1.006 g/ml fraction. As described for the cholesterol-fed dog, the  $\beta$ -VLDL were resolved into two fractions by gel filtration chromatography (Fig. 7). The first fraction eluted in the void volume, similar to Fraction I of dog  $\beta$ -VLDL; however, the second fraction eluted from the column somewhat earlier than observed for Fraction II of the cholesterol-fed dog (Fig. 1). The two fractions, pooled (horizontal bars) as shown in Fig. 7 for a representative study, were used for subsequent analyses.

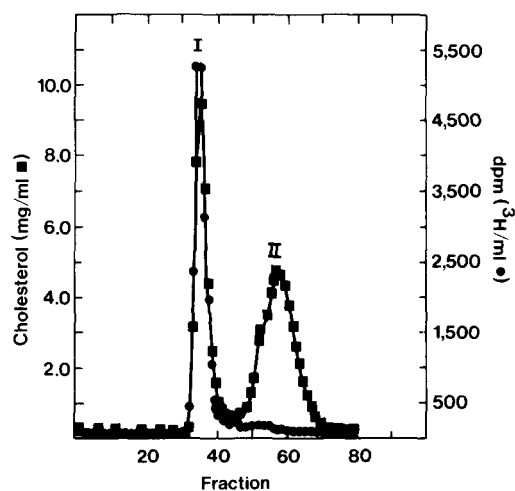


Fig. 5. Gel filtration chromatography of the ultracentrifugal d < 1.006 g/ml fraction of a hypercholesterolemic dog fed 300  $\mu$ Ci of [<sup>3</sup>H]retinol. Total cholesterol (■) and [<sup>3</sup>H]retinol (●) were measured on 3.2-ml column fractions. The A-15 m (4% agarose) column was run at 4°C and had a flow rate of 15 ml/hr.

Lipoprotein electrophoretograms of the  $\beta$ -VLDL and of the fractions obtained by gel filtration were similar to those observed for the cholesterol-fed dog. Fraction I remained at the origin, whereas Fraction II had  $\beta$ -electrophoretic mobility. Electron microscopic examination of these fractions revealed two major populations of particles (Fig. 8). In contrast to the dog  $\beta$ -VLDL fraction, the Fraction I lipoproteins from the Type III subjects were more homogeneous, spherical particles. In addition, the human Fraction I particles were smaller than the Fraction I particles from the dog (78 vs. 159.7 nm). The human Fraction II particles were slightly larger than the corresponding Fraction II particles in the dog (39.6 vs. 35.0 nm).

Further characterization of these fractions from the Type III subjects revealed several similarities to the corresponding fractions of  $\beta$ -VLDL in the dog. The chemical compositions and the apoprotein constituents were similar. As shown in Table 3, the total cholesterol to protein ratios of Fractions I and II were similar to those obtained for the canine fractions. Likewise, the apoprotein constituents of the human  $\beta$ -VLDL fractions closely resembled those of the dog. Fraction II of the human  $\beta$ -VLDL contained predominantly the higher molecular weight form of apo-B, whereas Fraction I possessed predominantly the lower molecular weight form (Fig. 9).

#### Effect of $\beta$ -VLDL and $\beta$ -VLDL fractions on cholesterol esterification in mouse peritoneal macrophages in culture

The  $\beta$ -VLDL and the two chromatographically isolated fractions of  $\beta$ -VLDL from both cholesterol-fed dogs

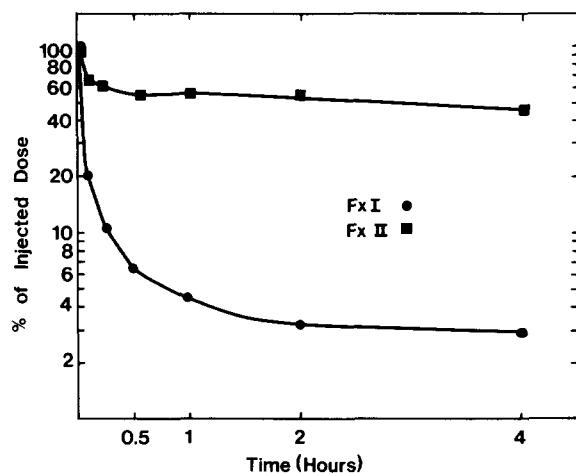


Fig. 6. Plasma clearance of  $^{131}\text{I}$ -labeled Fraction I and  $^{125}\text{I}$ -labeled Fraction II of canine  $\beta$ -VLDL injected into a cholesterol-fed dog (plasma cholesterol  $\sim 750$  mg/dl). The results are expressed as trichloroacetic acid-precipitable counts remaining in the plasma at each time interval. Approximately 0.5 mg of lipoprotein protein of both Fractions I and II were injected simultaneously.

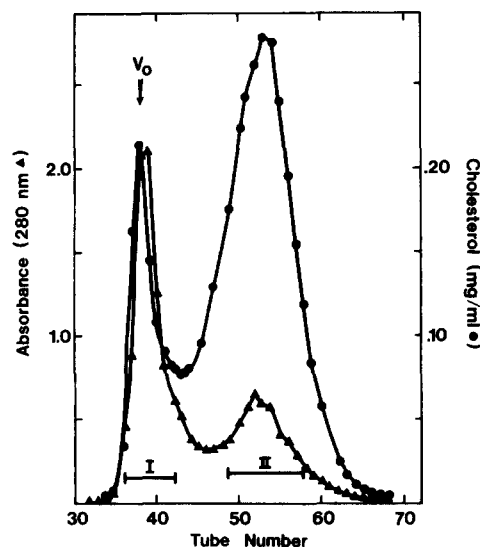


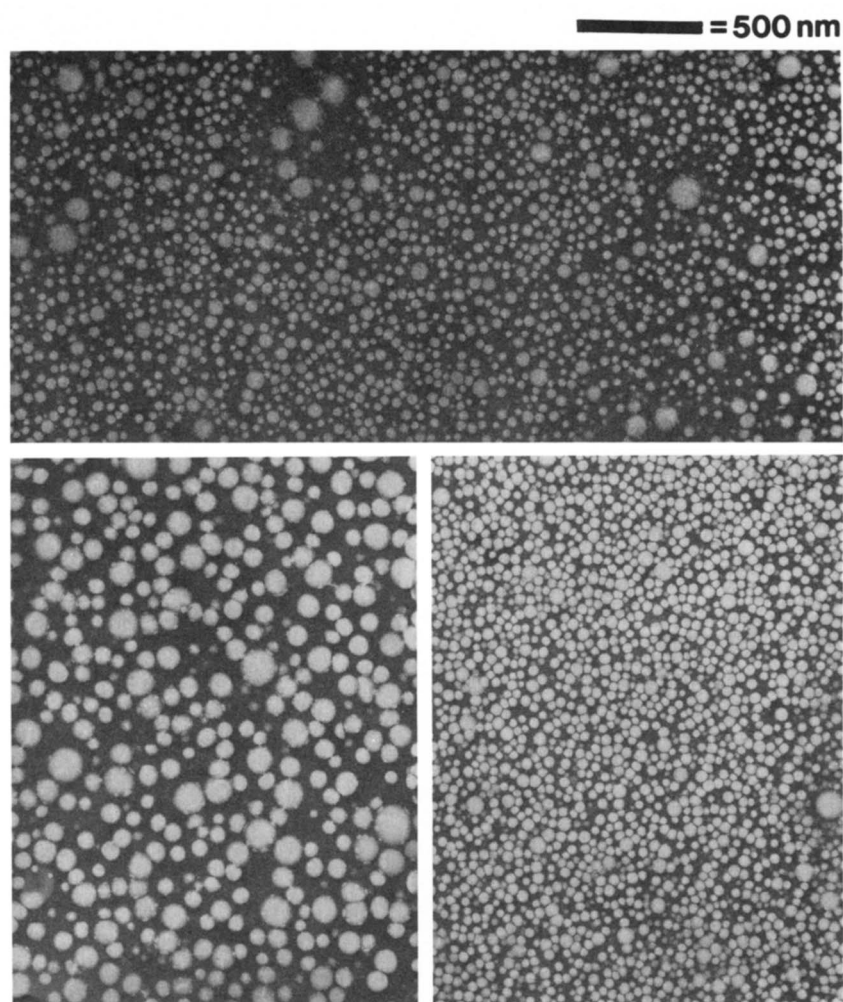
Fig. 7. Gel filtration permeation chromatography of human  $\beta$ -VLDL from a Type III subject (S.B.). The  $\beta$ -VLDL was isolated from plasma by Geon-Pevikon block electrophoresis from the  $d < 1.006$  g/ml fraction of plasma. The  $\beta$ -VLDL (3 mg of lipoprotein protein) was applied to the same column and operated under similar conditions as used for the study shown in Fig. 1 (the recovery for cholesterol was 92%). The two fractions were pooled and concentrated (bars).

and Type III hyperlipoproteinemic patients were capable of promoting cholesteryl esterification in mouse peritoneal macrophages (Table 4). Fraction I lipoproteins from both dogs and humans were most active in stimulating cholesteryl ester synthesis. When the lipoproteins were added to the cells at an equal lipoprotein cholesterol concentration, Fraction I lipoproteins from both dogs and humans were threefold more active than the Fraction II lipoproteins. When the dog  $\beta$ -VLDL fractions were added at an equal protein concentration (5  $\mu\text{g}/\text{ml}$ ), this difference was even more evident. Under these conditions, Fraction I was 17-fold more active than Fraction II in promoting cholesteryl esterification (Table 4).

Previously, we reported that the  $d < 1.006$  g/ml fraction from subjects with Type III hyperlipoproteinemia lacked the ability to stimulate cholesteryl ester synthesis in macrophages (27). As shown in Table 4 for subject D.R., it was difficult to demonstrate significant enhancement of cholesteryl ester synthesis by the unfractionated  $d < 1.006$  g/ml fraction. However, the  $\beta$ -VLDL, and especially Fraction I of the  $\beta$ -VLDL, were markedly active.

Previously, it has been shown that the whole  $d < 1.006$  g/ml fraction or the unfractionated  $\beta$ -VLDL from cholesterol-fed dogs stimulated cholesterol esterification in macrophages and led to cholesteryl ester accumulation (9, 10). These lipoproteins were taken up by a  $\beta$ -VLDL receptor or binding site, which was distinct from the modified LDL binding site (19). It was of interest to





**Fig. 8.** Electron micrographs of negatively stained human  $\beta$ -VLDL (S.B.) and its fractions separated by gel filtration (Fig. 5). Fraction I (lower left) and Fraction II (lower right) are shown. The inserted bar represents 500 nm. The mean particle diameter  $\pm$  S.D. and the median, respectively, are  $78.3 \pm 21.4$  and 77.5 nm for Fraction I and  $39.6 \pm 8.2$  and 36.7 nm for Fraction II.  $\times 33,000$ .

determine if both Fractions I and II were mediating their delivery of cholesterol to the macrophages via the  $\beta$ -VLDL receptors (9, 10). Fucoidin, a known inhibitor

of modified LDL uptake but not of  $\beta$ -VLDL uptake by macrophages (9, 10), was shown to inhibit the binding and degradation of acetoacetylated human LDL; however, fucoidin had only a minor effect on the binding and degradation of Fraction I, the most active fraction of the  $\beta$ -VLDL (**Fig. 10**). Similarly, fucoidin had little effect in inhibiting the binding and degradation of the fractions of  $\beta$ -VLDL obtained from the patients with Type III hyperlipoproteinemia (data not shown). Furthermore, Fractions I and II of the canine  $\beta$ -VLDL were incapable of significantly displacing acetylated human LDL, as determined in competitive binding and degradation assays performed with mouse peritoneal macrophages (**Fig. 11**). Thus, it appears that the interaction of both fractions with the macrophages was mediated predominantly by the  $\beta$ -VLDL receptors.

TABLE 3. Ratio of total cholesterol to protein in the  $\beta$ -VLDL fractions

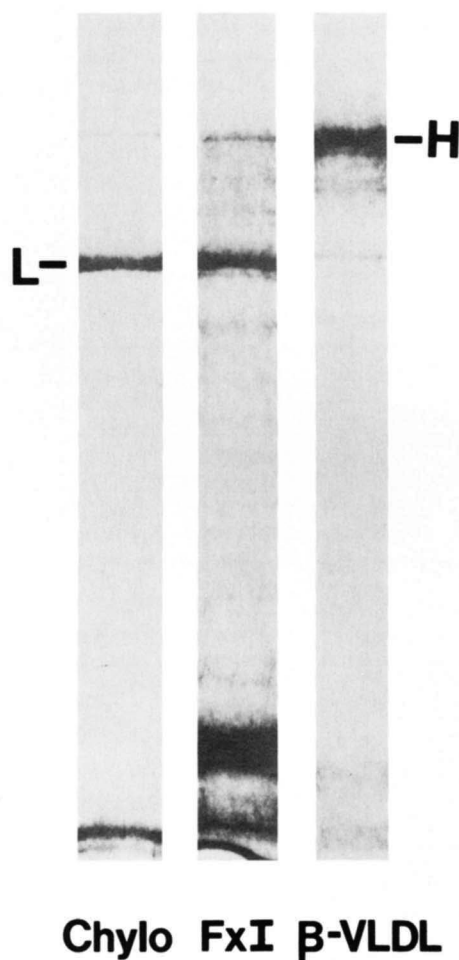
	Dog <sup>a</sup>	Human <sup>b</sup>	
		S.B.	D.R.
Fraction I	13.3	9.1	9.8
Fraction II	4.0	3.8	3.8

<sup>a</sup> Values for the ratios of total cholesterol to protein are derived from Table 1.

<sup>b</sup> Ratios of total cholesterol to protein in Fractions I and II of  $\beta$ -VLDL are from subjects (S.B. and D.R.) with Type III hyperlipoproteinemia.

## DISCUSSION

The  $\beta$ -VLDL are cholesteryl ester-rich lipoproteins that appear in the  $d < 1.006$  g/ml ultracentrifugal fraction of plasma from cholesterol-fed animals, including dogs (1, 2). The  $\beta$ -VLDL can be separated from pre- $\beta$  lipoproteins (VLDL) by Geon-Pevikon block electrophoresis. Up to now, we have considered the  $\beta$ -VLDL a homogeneous class of lipoproteins, and they have been studied as such (9, 10). In the present study, we demonstrated that  $\beta$ -VLDL represent a heterogeneous group of lipoproteins that can be subfractionated into two distinct fractions. The two fractions isolated from



**Fig. 9.** SDS-polyacrylamide slab gel electrophoresis performed on 5% gels. The unfractionated  $\beta$ -VLDL and the Fraction I lipoproteins from a subject (D.R.) with Type III hyperlipoproteinemia are compared. Fraction I (FxI) contains primarily the lower molecular weight form of apo-B which migrates in a position equivalent to the low molecular weight apo-B of chylomicrons. The  $\beta$ -VLDL and the Fraction II lipoproteins (not shown) contain predominantly the higher molecular weight form of apo-B. The higher molecular weight apo-B comigrates with the apo-B of normal plasma LDL.

**TABLE 4.** Stimulation of cholesteryl ester formation in mouse peritoneal macrophages by  $\beta$ -VLDL fractions of cholesterol-fed dogs and Type III hyperlipidemic patients<sup>a</sup>

Lipoprotein Fraction Added to Medium	Concentration of Lipoprotein in Medium		Cholesteryl [ <sup>14</sup> C]oleate Synthesis
	Protein	Cholesterol	
	$\mu\text{g/ml}$		$\text{nmol/mg}$ <i>cellular protein</i>
Dog lipoproteins			
Experiment I <sup>b</sup>			
None			0.37
$\beta$ -VLDL	9.3	50	7.4
Fraction I	3.7	50	14.6
	7.4	100	25.5
Fraction II	12.4	50	4.8
	24.8	100	10.1
Experiment II <sup>c</sup>			
None			0.37
Fraction I	5.0	66.7	30.6
Fraction II	5.0	20.2	1.8
Human lipoproteins (S.B.) <sup>b</sup>			
$\beta$ -VLDL	28.0	150	3.0
	56.0	300	4.3
Fraction I	11.2	150	23.5
	22.4	300	26.2
Fraction II	37.5	150	6.2
	75.0	300	5.1
Human lipoproteins (D.R.) <sup>d</sup>			
None			1.2
d < 1.006	59	100	2.0
VLDL	70	100	2.7
$\beta$ -VLDL	31	100	5.0
Fraction I	19	100	13.3
Fraction II	43	100	2.7

<sup>a</sup> Each monolayer (16-mm plastic Petri dishes) received 0.5 ml of DMEM containing 0.2 mM [<sup>14</sup>C]oleate with 2.4 mg/ml albumin (sp act 18,000 dpm/nmol) and the indicated concentration of the lipoprotein fractions (cholesterol-fed dog and human Type III [S.B.]  $\beta$ -VLDL and its fractions separated by gel filtration, Figs. 1 and 5). After incubation for 16 hr at 37°C, the cellular content of cholesteryl [<sup>14</sup>C]oleate was determined and the results expressed per mg of cellular protein.

<sup>b</sup> The studies with the dog lipoproteins (Experiment I) and the human Type III lipoproteins were performed with the same batch of cells on the same day. The lipoproteins were added at equal cholesterol concentrations.

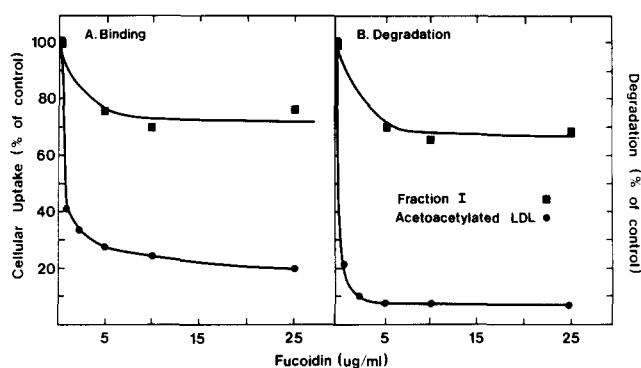
<sup>c</sup> In this experiment, cholesterol-fed dog lipoprotein fractions were added at equal lipoprotein concentrations.

<sup>d</sup> A separate experiment performed as described in Footnote a. The  $\beta$ -VLDL and VLDL (pre- $\beta$  lipoproteins) were isolated from the  $d < 1.006$  g/ml fraction by Geon-Pevikon block electrophoresis.

$\beta$ -VLDL by agarose column chromatography differed in size, morphology, electrophoretic mobility on paper, chemical composition, and apoprotein B forms. However, both fractions were rich in cholesteryl ester, had similar apoprotein compositions (B and E), and coelectrophoresed by Geon-Pevikon block electrophoresis.

The differences between these two fractions suggest





**Fig. 10.** Effects of the increasing concentration of fucoidin on the binding and internalization (A) and degradation (B) of acetoacetylated  $^{125}\text{I}$ -labeled LDL (●), and  $^{125}\text{I}$ -labeled Fraction I lipoproteins (■) from a cholesterol-fed dog. Unstimulated mouse peritoneal macrophage monolayers in culture were incubated with DMEM containing dog Fraction I (10 µg/ml) or human acetoacetylated LDL (10 µg/ml).

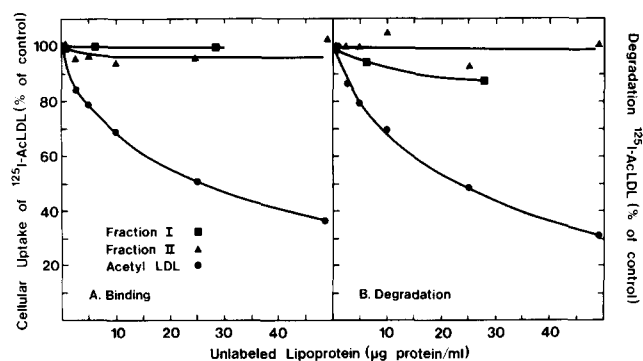
that they were of different origin. It appears that Fraction I was of intestinal origin and might represent a type of chylomicron remnant induced by the high cholesterol diet. The Fraction I lipoproteins were large particles (90–300 nm in diameter) with an irregular shape, resembling chylomicrons or their remnants. Furthermore, they contained relatively small amounts of protein (3.3% by weight) and possessed the lower molecular weight form of the B apoprotein. The lower molecular weight form of apo-B has been shown to arise, at least in part, from the intestine, and has been shown to be a principal apoprotein constituent of intestinal lipoproteins (23–25). Further evidence suggesting an intestinal origin for Fraction I was provided by the observation that this fraction could be markedly reduced by prolonged fasting and that this fraction was the principal constituent of the  $d < 1.006$  g/ml lipoproteins transporting orally administered  $[^3\text{H}]$ retinol. In addition, the plasma clearance of the Fraction I lipoproteins in cholesterol-fed dogs resembled the clearance of chylomicrons, whereas the plasma clearance of Fraction II lipoproteins was distinctly different. It is possible that this fraction can be further subfractionated.

It is reasonable to speculate that the Fraction II lipoproteins may be of hepatic origin. It has been shown that cholesterol-rich lipoproteins, resembling  $\beta$ -VLDL, were present in the Golgi apparatus (28) and secreted by the perfused liver (29) of cholesterol-fed rats. The presence of the high molecular weight form of the apo-B, and the fact that the concentration of the Fraction II lipoproteins was not decreased significantly by prolonged fasting, indicates that they were not of intestinal origin. Furthermore, it was previously shown that the  $d < 1.006$  g/ml lipoproteins of cholesterol-fed dogs did not arise as products of intestinal (lymph) lipoproteins (12). The Fraction II lipoproteins have a protein content resem-

bling VLDL (14.5% by weight) and a particle size similar to the particle size of VLDL (30–75 nm).

Thus, it appears that the  $\beta$ -VLDL, which accumulate in the plasma of cholesterol-fed dogs, are a mixture of two lipoproteins: one possibly originating in the intestine and resembling chylomicron remnants (Fraction I), and the other representing true  $\beta$ -VLDL, which are possibly synthesized by the liver (Fraction II). The  $d < 1.006$  g/ml fraction of cholesterol-fed dogs also contains a third lipoprotein, the pre- $\beta$ -migrating VLDL, which is similar to normal canine VLDL. To resolve the various components of this fraction, it was necessary to use a combination of Geon-Pevikon block electrophoresis (to separate the  $\beta$ - [ $\beta$ -VLDL] and pre- $\beta$ - [VLDL] migrating lipoproteins) and agarose column chromatography (to separate Fractions I and II of the  $\beta$ -VLDL). The VLDL were not discretely resolved from the two  $\beta$ -VLDL fractions by agarose column chromatography. It is important to note that these two fractions of  $\beta$ -VLDL were present in the plasma even after a 14- to 16-hr (overnight) fast.

The potential significance of the  $\beta$ -VLDL in atherosclerosis has been suggested previously (1, 2). The  $\beta$ -VLDL possess the unique ability to induce cholesteryl ester synthesis and accumulation in macrophages (9, 10). Comparison of the two fractions of  $\beta$ -VLDL obtained by gel filtration chromatography revealed that both fractions were capable of promoting cholesteryl ester synthesis in mouse peritoneal macrophages. However, Fraction I was severalfold more active than Fraction II, based either on equal lipoprotein cholesterol or on protein added to the cells. Previously, it has been shown that  $\beta$ -VLDL were taken up through a receptor-mediated process distinct from the modified LDL binding site (9,



**Fig. 11.** Ability of human acetyl LDL (●), dog Fraction I from  $\beta$ -VLDL (■), and dog Fraction II from  $\beta$ -VLDL (▲) to compete with human acetyl  $^{125}\text{I}$ -labeled LDL for binding and internalization (A) and degradation (B) by mouse peritoneal macrophages. One ml of DMEM containing 10% lipoprotein-deficient serum, 2 µg/ml of human acetyl  $^{125}\text{I}$ -labeled LDL, and the indicated concentration of unlabeled lipoproteins were added to 36-mm Petri dishes. The cells were incubated for 5 hr at 37°C after which the cellular uptake and degradation of the iodinated acetyl LDL were determined as described in Materials and Methods.



10). In the present study, the delivery of cholesterol to the macrophages by both Fractions I and II was mediated predominantly via the  $\beta$ -VLDL receptor, not the modified LDL binding site.

In studies of two subjects with typical Type III hyperlipoproteinemia (dysbetalipoproteinemia), we were able to isolate subfractions of  $\beta$ -VLDL. The Fractions I and II from the human Type III  $\beta$ -VLDL were similar in physical and chemical characteristics to the canine subfractions. Furthermore, it was shown that the Fraction I lipoproteins had an enhanced ability to stimulate cholesteryl ester synthesis in macrophages. The presence of more than one particle in the  $d < 1.006$  g/ml fraction of plasma of patients with Type III hyperlipidemia has been previously reported by Sata, Havel, and Jones (30) and by Packard et al. (31). The present study suggests that the subfractions are metabolically distinct and possibly of different origin.

In summary, it appears that the  $\beta$ -VLDL of the cholesterol-fed dog are comprised of two distinct subclasses of lipoproteins, one originating in the intestine and possibly representing chylomicron remnants, and the other possibly originating from the liver. The fraction of intestinal or chylomicron remnant origin, which is most active in stimulating cholesteryl ester synthesis in macrophages, may be of importance in atherogenesis. These results suggest that the findings in dogs may be relevant to patients with Type III hyperlipoproteinemia. ■

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