Effect of cholesterol feeding on the distribution of plasma lipoproteins and on the metabolism of apolipoprotein E in the rabbit[1]

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Abstract Changes in lipoprotein distribution and in apolipoprotein E metabolism were studied in rabbits fed a diet containing 1% cholesterol. Lipoprotein distribution was monitored by rate zonal ultracentrifugation, gel filtration chromatography, and electrophoretic techniques. Normolipidemic rabbit plasma contained VLDL, IDL, LDL, and HDL. Within 1 week on the 1% cholesterol diet, the d < 1.006 g/ml material was greatly elevated, and the lipoproteins of higher density (LDL and HDL) decreased below levels of detection. Cholesteremic d < 1.006 g/ml material was cholesteryl ester-rich, triglyceride-poor, and contained particles of S_f 20 to >400. Upon diet normalization, the LDL and HDL reappeared within 2–4 weeks accompanied by a decrease in the d < 1.006 g/ml material. The metabolism of apoE was studied by injecting purified and ^{125}I-labeled apoE into rabbits and following the clearance of the tracer. ApoE in the normolipemic rabbit demonstrated a fractional catabolic rate (FCR) of 0.132 hr^{-1} and a half-life ($t_{1/2}$) of 10.3 hr. ApoE in the hypercholesteremic rabbit demonstrated an FCR of 0.055 hr^{-1} and a $t_{1/2}$ of 49.5 hr. ApoE concentrations in the plasma as estimated by electroimmunoassay were 19.5 mg/dl in the control rabbit and 199 mg/dl in the hypercholesterolemic rabbit. From these data, absolute synthetic rates of 20.3 mg/kg per day and 86.1 mg/kg per day were calculated for the control and the hypercholesterolemic rabbit, respectively. We conclude that the cholesterol-supplemented diet caused pronounced elevation of apoE in the plasma due to overproduction of the protein.—**Roth, R. I., J. W. Gaubatz, A. M. Gotto, Jr., and J. R. Patsch.** Effect of cholesterol feeding on the distribution of plasma lipoproteins and on the metabolism of apolipoprotein E in the rabbit. *J. Lipid Res.* 1983. **24:** 1–11.

Supplementary key words "arginine-rich" protein • fractional catabolic rate

The relationship between dietary lipid and atherosclerosis has been a subject of extensive investigation over the last six decades. Early work by pathologists as summarized by Duff (2) established the cholesterol-fed rabbit as a useful experimental model to study this relationship. Cholesterol added to the diet in an abundance of 0.5–2% has been shown to result in a rapid development of hypercholesterolemia (3–5) and subsequently in the appearance of atheromata (6, 7). Plasma triglyceride levels have been reported to increase or remain normal (8–10) or decrease (11) in response to dietary cholesterol. During the past 20 years, a major focus of interest has become the characterization of cholesterol-induced changes in the rabbit plasma lipoproteins. Gofman et al. (12) described an initial elevation of normal lipoproteins with S_f 5–8 followed by the appearance of new lipoproteins with S_f 10–30. His data indicated a positive correlation between the extent of atherosclerosis and plasma levels of intermediate density lipoproteins (S_f 10–30) but not with low density lipoproteins (S_f 5–8). Schumaker (3) reported that the S_f 0–400 material, which was greatly elevated in cholesterol-fed rabbits, was relatively rich in cholesteryl esters and poor in triglycerides compared to the corresponding lipoprotein fraction of control rabbits. In a subsequent investigation, Camejo et al. (9) demonstrated that these cholesterol-induced changes affected primarily the d < 1.019 g/ml material which corresponds to lipoproteins of S_f 12–400.

Shore, Shore, and Hart (4) confirmed the vast elevation of VLDL (20- to 44-fold) induced by cholesterol feeding and noticed a 4- to 5-fold increase in LDL_1 and

Abbreviations: S_f, rate of flotation at d 1.063 g/ml, 26°C, expressed in Svedbergs; VLDL, very low density lipoproteins (d 1.006 g/ml; S_f 20–400); CER-VLDL, β-VLDL, cholesteryl ester-rich VLDL from cholesterol-fed rabbits; IDL, intermediate density lipoproteins (d 1.006–1.019 g/ml, S_f 12–20); LDL, low density lipoproteins (d 1.019–1.063 g/ml, S_f 0–12); HDL, high density lipoproteins (d 1.063–1.210 g/ml); HDL_c, cholesterol-induced animal lipoproteins found at d 1.02–1.09 g/ml; apoE, "arginine-rich" protein, protein component of VLDL, IDL, and HDL; apoB, major protein component of chylomicrons, VLDL, IDL, and LDL; apoA-I, major protein component of HDL; apoA-II, major protein component of human HDL; EDTA, ethylenediaminetetraacetic acid; Tris, Tris(hydroxymethyl)-aminoethane; SDS, sodium dodecyl sulfate; FCR, fractional catabolic rate; LCAT, lecithin:cholesterol acyltransferase.

[1] A portion of this work has appeared in abstract form (1).

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LDL_2 that was accompanied by a reduction in HDL concentration. They found that the VLDL were larger than their normal counterparts as determined by electron microscopy, had β-mobility in agarose gel electrophoresis, and were specifically enriched in apoE (to about 50% of the VLDL protein). Stange, Agostini, and Papenberg (8) observed HDL with altered composition in structure. In contrast to the report by Shore et al. (4), Pinon and Bridoux (13) reported that after rabbits had been on a 1% cholesterol diet for 12 weeks, plasma levels of HDL had not changed, but the HDL unesterified cholesterol increased from 2.3% to 18.5% and cholesteryl esters decreased from 15.5% to 5%.

In this study, lipoprotein distributions of control and cholesterol-fed rabbits were compared with the use of zonal ultracentrifugation. Alteration of lipoprotein distribution was confirmed by lipoprotein electrophoresis and gel filtration chromatography. Cholesterol feeding increased the plasma concentration of cholesterol and apoE. In an effort to understand whether the rise in apoE plasma levels was brought about by overproduction or impaired catabolism, absolute synthetic rate and fractional catabolic rate of apoE were estimated.

MATERIALS AND METHODS

Dietary management

Adult female New Zealand white rabbits weighing 2–3 kg were maintained on a commercial rabbit chow (Purina) or on a diet supplemented with 1% cholesterol (Sigma, St. Louis, MO). The cholesterol-supplemented diet was prepared by dissolving cholesterol in ether, spraying the solution onto the rabbit chow, and then allowing the ether to evaporate for at least 12 hr. Lipoprotein changes were studied in the postabsorptive state. For in vivo turnover studies, rabbits weighing 2.2–2.3 kg were used. To assure steady state conditions, the animals were maintained on either a normal diet or a diet supplemented with 1% cholesterol from 4 weeks prior to and throughout the study. For these studies, the animals were allowed to feed ad libitum regardless of the schedule of blood sampling.

Lipoprotein isolation

Blood was collected in 1% EDTA by cardiac puncture. VLDL was isolated from plasma by ultracentrifugation at plasma density for 18 hr at 55,000 rpm and 4°C in a Beckman L265B ultracentrifuge using a Beckman 40.3 rotor. LDL was isolated at d 1.063 g/ml by centrifuging at 55,000 rpm for 18 hr. HDL was subsequently isolated from the d 1.063 g/ml infranatant after its adjustment to d 1.210 g/ml and centrifugation at 55,000 rpm for 40 hr. For gel filtration chromatography, the total lipoprotein fraction was isolated from plasma by ultracentrifugation at d 1.210 g/ml, 55,000 rpm, and 4°C for 40 hr. Lipoproteins were dialyzed against a standard buffer containing 150 mM NaCl, 10 mM Tris, 1 mM EDTA, and 1 mM sodium azide, pH 7.4. In addition to sequential ultracentrifugation, lipoproteins were isolated by zonal ultracentrifugation using Beckman Ti-14 rotors. Analyses of VLDL were performed in a linear sodium bromide gradient (d 1.0–1.15 g/ml) at 42,000 rpm, 14°C for 45 min (14). Total VLDL, IDL, and LDL were analyzed using a linear NaBr gradient (d 1.00–1.30 g/ml) at 42,000 rpm and 14°C for 140 min (15). HDL was analyzed using a nonlinear NaBr gradient (d 1.00–1.40 g/ml) at 41,000 rpm and 10°C for 22 hr (16).

In addition to zonal ultracentrifugation, lipoprotein distribution in plasma was analyzed by gel filtration chromatography using a BioGel A-5M column (1.6 × 100 cm) (17). The d < 1.21 g/ml plasma fraction was applied and eluted at 22°C in the standard buffer described above. Stokes radii (d < 1.006 g/ml lipoproteins) were estimated by analytical gel filtration (18) on a calibrated column of Sepharose 2B (0.9 × 100 cm) in the standard buffer at 4°C as described previously (19). Lipoprotein electrophoresis of whole serum or isolated lipoproteins was performed on paper strips (20) on Geon-Pevikon blocks (21), and on agarose gel (22).

Purification of apoE

ApoE was isolated from the VLDL of cholesterol-fed rabbits after delipidation of VLDL with diethyl ether–ethanol 3:1 and subsequent chromatography of apoVLDL on Sephadex G-200 in 8 M guanidine hydrochloride as described previously (23). The purity of the isolated apoE was demonstrated by single bands on polyacrylamide gel electrophoresis in SDS and urea, and by double diffusion on Ouchterlony plates employing monospecific antibodies. Amino acid analyses were performed using a Beckman model 119 analyzer.

Polyacrylamide gel electrophoresis

Apolipoproteins were solubilized in 50% tetramethylurea (24) and analyzed by electrophoresis using 7.5% polyacrylamide gels containing 0.1% SDS or 8 M urea at pH 8.4.

Chemical analyses

Lipoproteins were analyzed for protein by the method of Lowry et al. (25) using bovine serum albumin as a standard and for phospholipids by the method of Bartlett (26) using a factor of 25 to convert inorganic phosphorus to phospholipid. In plasma and in isolated lipoprotein fractions, triglycerides were estimated using

a Technicon autoanalyzer (27) and free cholesterol and cholesteryl esters by an enzymatic procedure (28). To estimate LCAT activity, initial cholesterol esterification rates in plasma were determined using an enzymatic procedure (29).

Protein labeling

Purified apoE was labeled with ^{125}I according to a modification (30) of the McFarlane procedure (31). To remove excess iodine from the protein, the labeling mixture was chromatographed on Sephadex G-25. The molar ratio of iodine to protein was below 1.0.

In vivo studies

Metabolism of apoE in vivo was studied by injecting purified ^{125}I-labeled apoE into the circulation of the animals through a lateral ear vein and then taking periodic blood samples for total plasma radioactivity decay and for lipoprotein distribution of the tracer protein. Thyroidal uptake of iodine was prevented by the administration of KI (0.1 g/l) to the animals via the drinking water 1 day prior to and throughout the study. Blood (5–10 ml) was collected by cardiac puncture at appropriate intervals. Radioactivity was monitored using a Packard Autogamma spectrometer. Decay curves proved to be biexponential and were processed by the mathematical procedure of Matthews (32) to determine the fractional catabolic rate (FCR) and the apparent half-life of catabolism ($t_{1/2}$) from the terminal exponential, designated exponential I. Exponential I, extrapolated back to 0 time, was subtracted from the actual decay curve yielding a single exponential (designated exponential II). ApoE was quantified in frozen serum samples from control and cholesterol-fed rabbits using rocket immunoelectrophoresis (33) and an antiserum raised in a goat against rabbit apoE. The antiserum was monospecific against rabbit apoE and did not react with rabbit apoB or rabbit apoA-I. Electrophoresis was performed at 10°C at 3.0 v/cm, constant current setting for 12 hr. Veronal buffer (0.05 M, pH 8.6) was used in preparation of agarose, for serum and standard dilution, and in the electrophoresis cell chambers. The assay was linear over the range 18–102 μg apoE standard/ml ($r > 0.95$). The within-batch coefficient of variation was 4.3% (n = 36). Pool sizes were calculated as the product of apoE plasma concentration and the plasma volume which averages 3.28% of body weight in females of this rabbit strain (34). The product of the pool size times the FCR obtained from the plasma decay curves gave the absolute catabolic rate (ACR) which, under steady-state conditions, equals the synthetic rate (SR). The rabbits maintained constant weights and consumed a constant composition diet during the course of the metabolic studies.

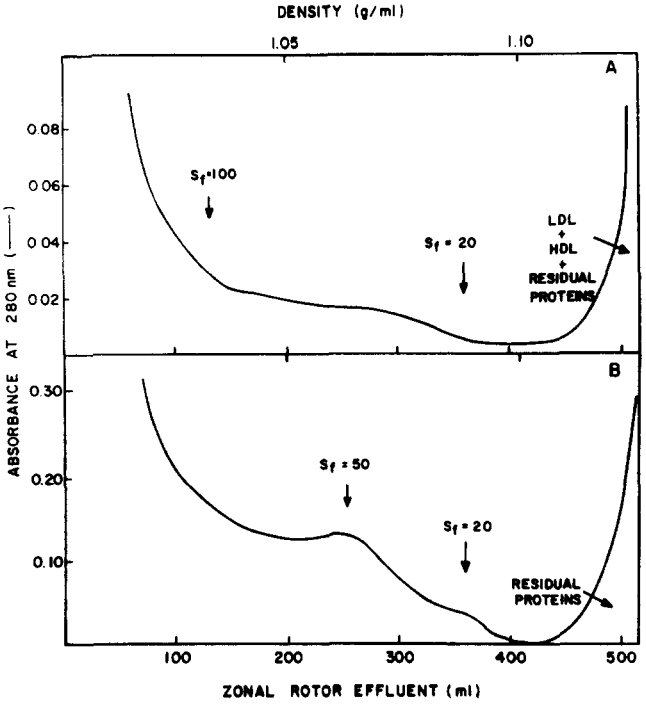

Fig. 1. VLDL analysis in the zonal rotor of normolipidemic plasma (A) and hypercholesterolemic plasma (B). Rabbit plasma samples analyzed were 10 ml. Ultracentrifugation was performed in a linear NaBr gradient in the density range 1.00–1.15 g/ml for 45 min (14). Arrows indicate the elution volumes for lipoproteins with the indicated S_f rates. Only the first 500 ml of the zonal rotor (665 ml total) are shown.

RESULTS

Rabbit lipoproteins in the normal and hypercholesterolemic state

Five fasted control rabbits used in our experiments had cholesterol concentrations ranging from 80–105 mg/dl and triglyceride levels of 45–83 mg/dl. The VLDL-cholesterol:total plasma triglyceride ratio averaged 0.14. Upon cholesterol feeding for 4 weeks or longer, plasma cholesterol concentrations ranged from 1,000–1,500 mg/dl and plasma triglyceride levels from 40–52 mg/dl. The VLDL cholesterol:total plasma triglyceride ratio was always greater than 10. LCAT activity, monitored as the initial esterification rate of plasma free cholesterol, was 50 nmol/ml per hr in a control rabbit and 274 nmol/ml per hr in a cholesterol-fed animal. Lipoprotein electrophoresis on paper strips and agarose gel showed that postabsorptive control rabbit plasma contained β-, preβ-, and α-lipoproteins. In contrast, the lipoprotein electrophoresis pattern of the plasma of cholesterol-fed rabbits showed that the lipoprotein material had migrated to the β-position or remained at the origin; no α-lipoproteins were detected. Zonal ultracentrifugation was performed on the control and cholesterol-fed rabbit plasmas. **Fig. 1** shows the effluent patterns after zonal ultracentrifugation for VLDL

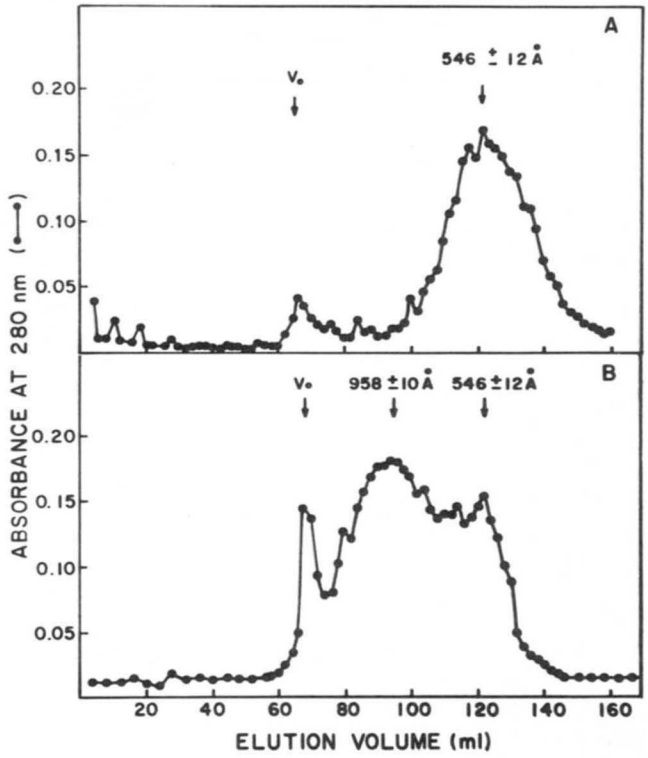

Fig. 2. Gel filtration chromatography of normal rabbit VLDL (A) and CER-VLDL (B) on a calibrated column (19) of Sepharose 2B equilibrated and eluted with standard buffer at 4°C. Stokes diameters were calculated (18) from elution volumes of 122 ml for normal VLDL and 94 ml for the larger component of cholesteremic VLDL. Two ml of each plasma sample was chromatographed. One of two experiments.

from normal rabbit plasma (Fig. 1A) compared to CER-VLDL[3] from hypercholesterolemic plasma (Fig. 1B). Both types of VLDL consisted of material spanning the full range of S_f 20–400, although CER-VLDL demonstrated a pronounced maximum at S_f 50–60 which was not present in normal VLDL. When compared by chromatography on a calibrated Sepharose 2B column, the normal VLDL (**Fig. 2A**) eluted mostly as a relatively symmetrical peak containing particles with a mean Stokes diameter of 546 Å; VLDL from the cholesterol-fed animals (Fig. 2B) was much more heterogeneous. Some material of 546 Å Stokes diameter was still present in the hypercholesterolemic rabbit, but a substantial quantity of particles was considerably larger with a mean Stokes diameter of 958 Å. When analyzed by Geon-Pevikon block electrophoresis, VLDL and LDL from control rabbits migrated at pre-β and β-migration rates, respectively. CER-VLDL exhibited a migration rate slower than that of normal VLDL, i.e., between β and pre-β (**Fig. 3**). In addition, cholesterol feeding resulted in a dramatic decrease in the abundance of triglycerides in these particles from ~60% (w/w) to ~2%, with a reciprocal increase in cholesteryl esters from ~2% to ~75% (**Table 1**).

The plasma zonal profile of normal rabbit VLDL, IDL, and LDL is shown in **Fig. 4A.** As evidenced by this figure, LDL is readily observed. When plasma from the cholesterol-fed rabbit was similarly analyzed, material emerged in the IDL range but LDL was entirely absent (Fig. 4B). This result was observed in four different rabbits on cholesterol-rich diets of between 4 weeks and more than 12 weeks.

Plasma HDL zonal patterns for normal and cholesterol-fed rabbits differed markedly (**Fig. 5**). Normal rabbits exhibited an apparently heterogeneous HDL peak (Fig. 5A) whose composition is given in Table 1. For apoprotein analysis, this peak was divided into three fractions, I, II, and III. In the cholesterol-fed animals, HDL was not detectable in the gradient by monitoring

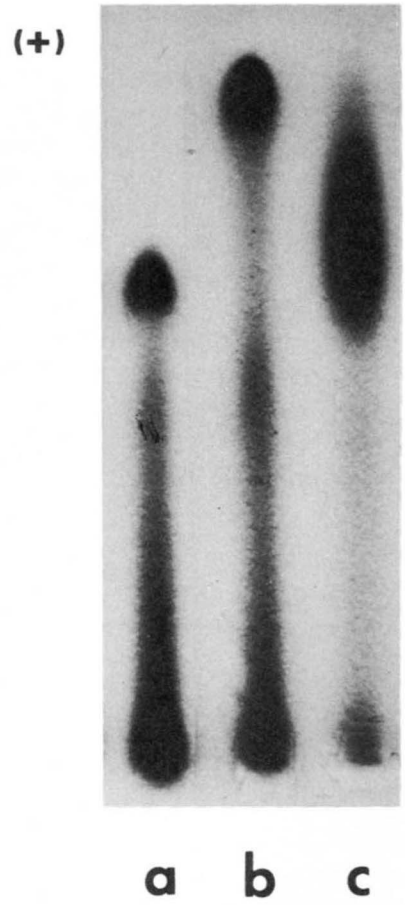

Fig. 3. Electrophoresis of (a) LDL, (b) VLDL from a control animal, and (c) CER-VLDL from a cholesterol-fed rabbit on Geon-Pevikon (21).

[3] It should be borne in mind that the abbreviation VLDL is strictly an operational notation referring to lipoprotein material whose composition confers a hydrated density of <1.006 g/ml. Clearly, lipoprotein particles may have entirely different proportions of lipid and/or protein constituents but similar or equivalent densities. This is illustrated in the present study by comparison of normal VLDL that is rich in triglyceride (about 62%) and poor in cholesteryl ester (about 2.2%) and hypercholesterolemic VLDL which contains only about 2% triglyceride but 73% cholesteryl ester.

optical density at 280 nm (Fig. 5B). Lipoprotein material eluting between 50 and 90 ml of the gradient, indicated by the hatched bar in Fig. 5B, was recentrifuged in a shallower gradient (d 1.0–1.3 g/ml) for VLDL–IDL–LDL, and was shown to contain only VLDL and IDL with no detectable LDL or HDL (data not shown). Chromatography on BioGel A-5M confirmed the data obtained by zonal ultracentrifugation. Gel filtration chromatography displayed peaks corresponding to VLDL, LDL, and HDL (**Fig. 6A**). In cholesterolemic plasma, gel filtration chromatography eluted only one lipoprotein fraction in the void volume corresponding to VLDL (Fig. 6B). Also, HDL was not detected by paper electrophoresis using a lipid stain (22), nor by agarose gel electrophoresis using precipitation (35) for visualization of lipoprotein bands.

Apoprotein composition of lipoproteins

Polyacrylamide gel electrophoresis in SDS indicated a greatly increased proportion of apoE in CER-VLDL as compared to VLDL from control rabbits that contained proteins migrating similar to human apoB and apoC (**Fig. 7A**). LDL contained only proteins migrating like apoB (data not shown). All three fractions I, II, and III from the heterogeneous HDL peak (Fig. 5A) contained almost exclusively a tetramethylurea-soluble apolipoprotein with electrophoretic mobility of human apoA-I (Fig. 7D). They also showed small amounts of albumin. None of the HDL fractions exhibited a protein band analogous to human apoA-II, in agreement with other reports (36).

Lipoprotein distribution in response to cholesterol feeding and reversibility

Because of drastic reduction of LDL and HDL in the plasma of cholesterol-fed rabbits, it was of interest to monitor changes in lipoprotein distribution during cholesterol feeding, and then during a subsequent period when cholesterol was withdrawn and the animals re-

TABLE 1. Percentage composition of rabbit plasma lipoproteins in the normolipidemic and hypercholesterolemic state

	Normolipidemic[a]			Hypercholesterolemic[b]	
	VLDL	LDL	HDL	CER-VLDL	CER-VLDL
Protein[c]	7.2	22.6	35.1	4.7	4.1
PL	23.6	25.6	36.0	11.0	10.9
UC	5.5	5.5	3.6	9.2	8.2
CE	2.2	23.8	16.2	73.2	75.2
TG	61.5	22.5	9.1	1.9	1.6

[a] Lipoproteins were isolated from pooled plasma of five normolipidemic rabbits.

[b] Two different rabbits on cholesterol-supplemented diet were used for isolation of CER-VLDL.

[c] PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triglyceride.

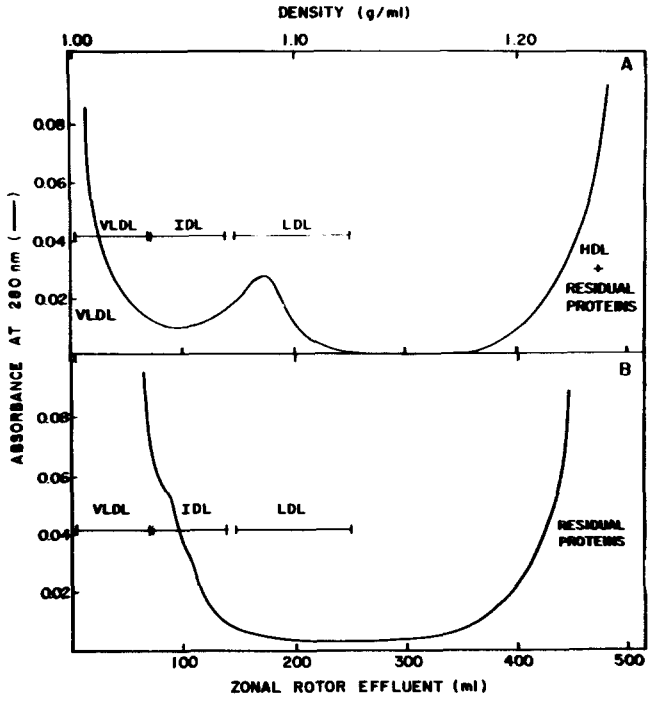

Fig. 4. Zonal effluent patterns of normal (A) and hypercholesterolemic (B) plasmas spun for IDL-LDL. Ten ml of normal plasma and 15 ml of hypercholesteremic plasma were centrifuged in a NaBr gradient d 1.00–1.30 g/ml for 140 min at 15°C (15). Bars indicate the elution volumes for human VLDL, IDL, and LDL.

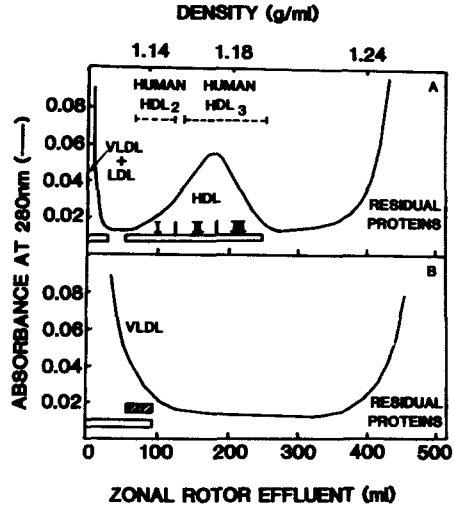

Fig. 5. Zonal rotor effluent patterns of HDL from the plasmas of normal (A) and cholesterol-fed (B) rabbits. Ten-ml samples were spun in a nonlinear NaBr gradient d 1.00–1.40 g/ml for 22 h at 10°C (16). Open bars indicate the fractions pooled for chemical analyses. With normolipidemic plasma, 92.4% of total plasma cholesterol was recovered in the VLDL + LDL and HDL fraction (panel A). With hypercholesterolemic plasma, 91.3% of total plasma cholesterol was recovered in the VLDL fraction (panel B). The dashed lines indicate the elution volumes for normal human HDL_2 and HDL_3. Material eluting in the area indicated by the hatched bar of panel B was pooled for subsequent recentrifugation in a shallower gradient (15) and found to consist of VLDL and IDL only (data not shown). Aliquots of gradient volume I, II, and III of panel A were used for apoprotein analysis by SDS gel electrophoresis (Fig. 7, D).

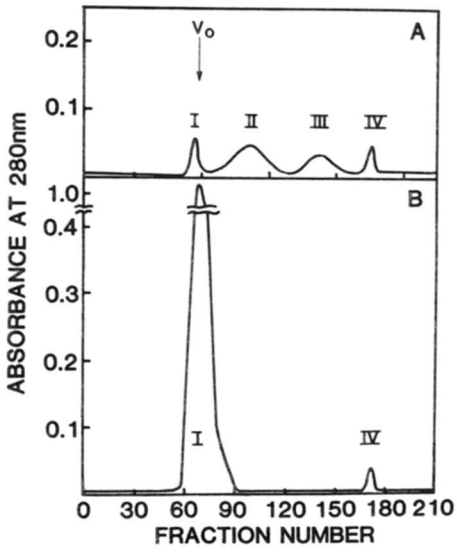

Fig. 6. Gel permeation chromatography of d < 1.210 g/ml ultracentrifugal fraction from identical plasma volumes of normal rabbit plasma (A), and hypercholesterolemic plasma (B). Using zonal ultracentrifugation and agarose gel electrophoresis, peaks I, II, III, and IV of panel A were identified as VLDL, LDL, HDL, and lipid-poor protein fractions, respectively. Using the same criteria for panel B, peak I exhibited VLDL-like flotation and LDL-like electrophoretic behavior; peak IV of panel B exhibited the same characteristics as peak IV of panel A.

turned to the control diet. **Fig. 8** shows the LDL zonal effluent patterns of plasma samples taken from a rabbit at various time intervals following the initiation of the 1% cholesterol diet. At 3 days, the rabbits had developed a moderate hypercholesterolemia (377 mg/dl), and LDL had been greatly reduced (Fig. 8A). Eight days after initiation of cholesterol feeding, LDL had virtually disappeared (Fig. 8B). **Fig. 9** shows the HDL zonal effluent patterns during the same period of cholesterol feeding. At 3 days (Fig. 9A), HDL was greatly reduced and at 8 days (Fig. 9B), 4.5 weeks, and at 7.5 weeks (Fig. 9C), HDL remained undetectable. After 7.5 weeks, the rabbit was returned to a diet of commercial chow without added cholesterol. **Fig. 10** shows the LDL zonal elution profiles of plasma samples taken from the same rabbit following return to the normal diet. After 2 weeks on the normal diet, mostly VLDL and IDL, but no LDL with typical flotation behavior were observed (Fig. 10A). After 4.5 weeks, LDL had fully reappeared (Fig. 10B). The normalization of HDL was also observed. After 8 days (**Fig. 11A**) and 2 weeks (Fig. 11B), HDL still had not increased to a significant level. By 4.5 weeks (Fig. 11C), HDL was restored to easily detectable levels; at this time, however, HDL of higher density still appeared somewhat reduced so that the HDL of lower density was relatively more abundant. At 7.5 weeks, the heterogeneity of HDL was still apparent and was similar to that prior to cholesterol feeding (Figs. 11D and 5A).

Metabolism of apoE

As described above, hypercholesterolemic rabbits contained almost exclusively d < 1.019 g/ml lipopro-

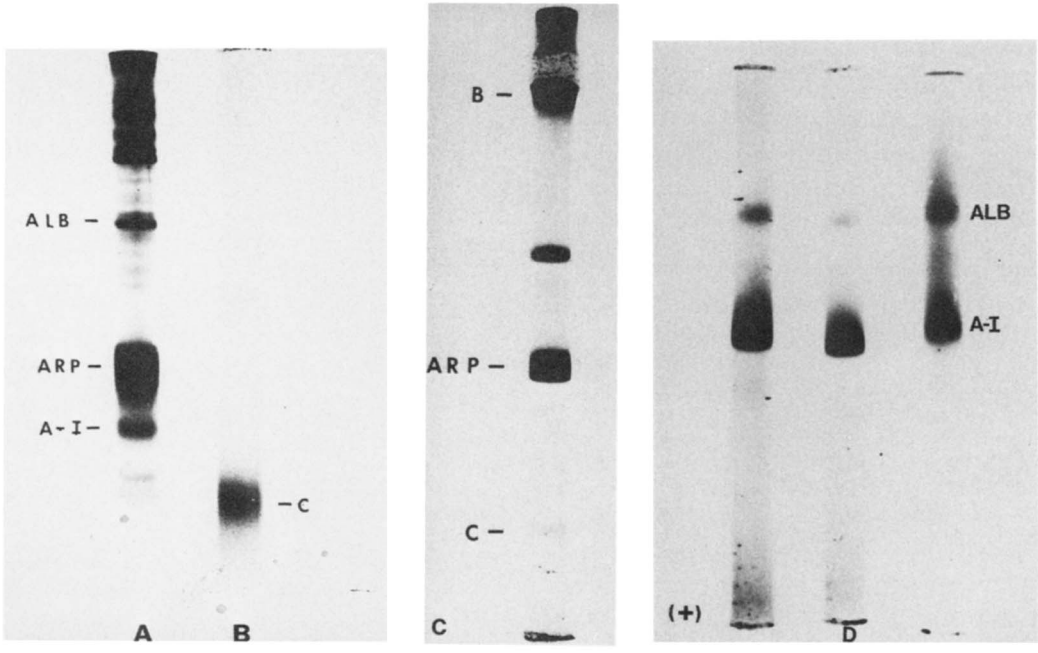

Fig. 7. Polyacrylamide gel electrophoresis in 0.1% SDS of apolipoproteins of a cholesterol-fed and normolipidemic rabbit. A, CER-VLDL; B, normolipidemic VLDL; C, IDL of cholesterol-fed rabbit; D, HDL of normolipidemic rabbit. The three gels contain (from left to right) the HDL gradient regions I, II, and III of Fig. 5A.

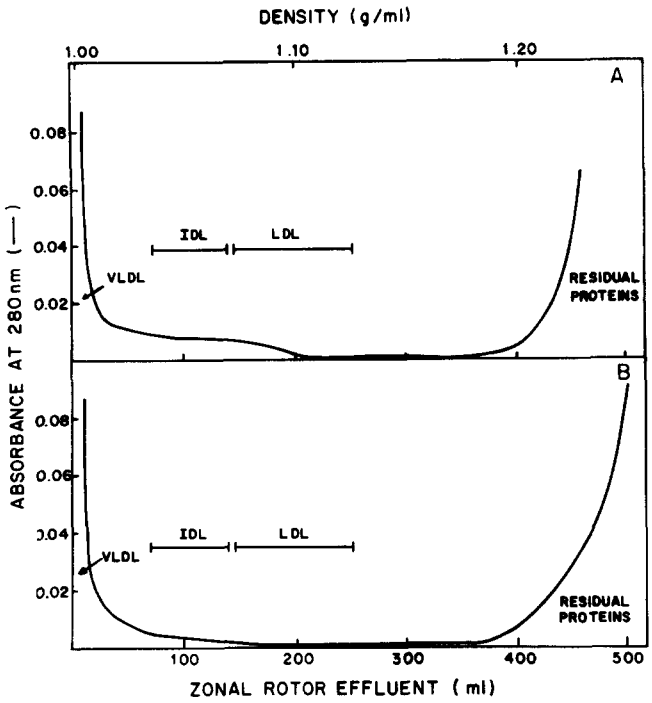

Fig. 8. Zonal rotor effluent patterns of IDL-LDL from rabbit plasma samples obtained 3 days (A) and 8 days (B) following initiation of a diet enriched with 1% cholesterol. Plasma aliquots were 4 ml. Experimental details are the same as described in the legend for Fig. 4.

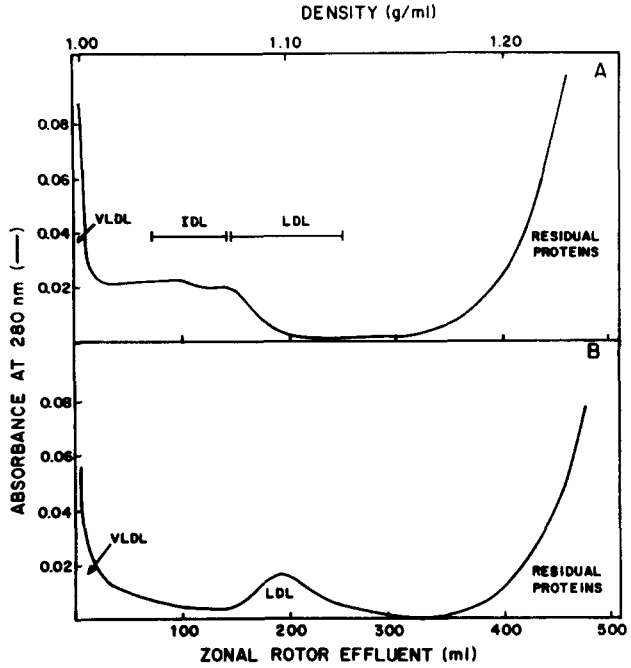

Fig. 10. Zonal profiles of rabbit plasma spun for IDL-LDL. Plasma samples were obtained 2 weeks (A) and 4.5 weeks (B) following cessation of the cholesterol-enriched diet. Each plasma sample was 4 ml. Bars indicate the elution volumes of rabbit IDL and LDL. Experimental conditions are the same as those described in the legend for Fig. 4.

tein material in their plasma. It was reported by Shore et al. (4) and confirmed in this work that the compositional differences in this material (high cholesterol vs. low triglyceride) are accompanied by a dramatic increase in the relative content of apoE. The high levels of apoE-enriched lipoprotein material resulted in an elevation of the total plasma apoE concentration. The

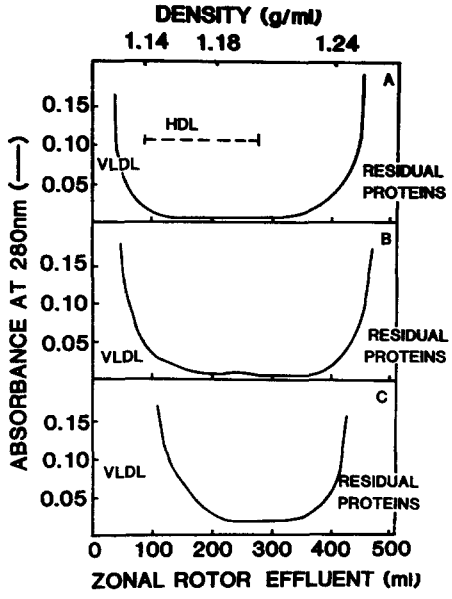

Fig. 9. Zonal effluent profiles of HDL subfractions from rabbit plasma obtained 3 days (A), 8 days (B), and 7.5 weeks (C) following initiation of the 1% cholesterol diet. Ten-ml plasma samples were centrifuged. The bars indicate the elution volumes of rabbit HDL. Experimental details are the same as those described in the legend for Fig. 5.

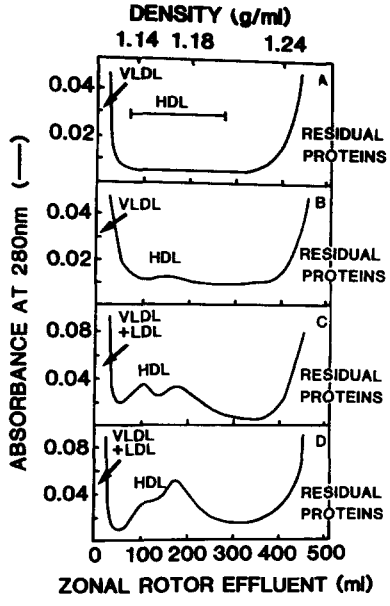

Fig. 11. Zonal effluent patterns of HDL from rabbit plasmas obtained 8 days (A), 2 weeks (B), 4.5 weeks (C), and 7.5 weeks (D) following cessation of the cholesterol-enriched diet. Samples were each 10 ml. Bar indicates the elution volume of rabbit HDL. Experimental details are the same as those described in the legend to Fig. 5.

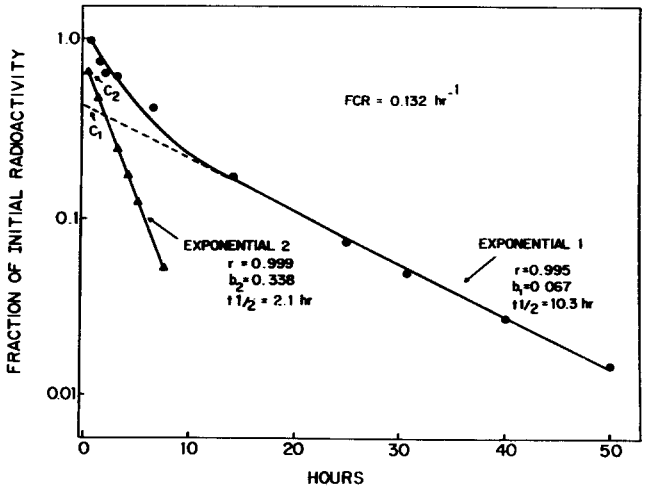

Fig. 12. Decay of plasma ^{125}I-labeled apoE in a normal rabbit; 3.5×10^6 cpm of ^{125}I-labeled apoE in 1.2 ml of the standard buffer was injected.

mechanism for this elevation in plasma apoE is unknown. It is possible that it reflects an increased synthesis or, alternatively, a blockage in the catabolism of the proteins. To investigate these possibilities, an in vivo turnover study was performed. ApoE was isolated from the d < 1.006 g/ml lipoprotein material of a cholesterol-fed rabbit, purified to homogeneity, and radiolabeled with ^{125}I, as described in the Methods section. ^{125}I-Labeled apoE was injected intravenously into rabbits, plasma samples were taken at short intervals, and the radioactivity was counted in plasma aliquots. In a control rabbit, ^{125}I-labeled apoE was cleared from the circulation with a biexponential decay (**Fig. 12**). The decay profile was resolved into two exponentials, yielding excellent correlation coefficients. Exponential I ($r = 0.995$), the terminal linear portion of the decay curve, reflected the clearance of ^{125}I-labeled apoE from the plasma. A fractional catabolic rate (FCR) of 0.132 hr^{-1} was calculated for this process, and the half-life ($t_{1/2}$) was 10.3 hr. Exponential II ($r = 0.999$) reflected the rate of equilibration of ^{125}I-labeled apoE between the intravascular and extravascular compartments. The half-time of the equilibration process was 2.1 hr.

Clearly altered metabolic parameters were found in the hypercholesterolemic state. The clearance of ^{125}I-labeled apoE from the plasma of a cholesterol-fed rabbit is shown in **Fig. 13.** This decay curve was also biexponential, and could be resolved into the terminal exponential I ($r = 0.993$) and into exponential II ($r = 0.993$). The half-time of equilibration obtained from exponential II was 2.6 hr, very similar to that of the normal rabbit (2.1 hr). However, in the cholesteremic state, only 5.5% of the total apoE was cleared from the circulation per hour (FCR = 0.055), whereas 13.2% of the apoE was cleared in the same time in the normolipemic state. The half-life was increased by a factor of five ($t_{1/2}$ = 49.5 hr). The apoE plasma concentration, as estimated by electroimmunoassay, was 199 mg/dl for the cholesterol-fed rabbit used in the turnover study. The apoE level in the control rabbit used in the turnover study was 19.5 mg/dl of plasma. The absolute catabolic rate (ACR), which, under steady-state conditions equals the absolute rate of synthesis, was 86.1 mg/kg per day for the cholesterol-fed animal and 20.3 mg/kg per day for the normal rabbit. Thus, the cholesterol-fed rabbit synthesized and cleared approximately four times more apoE per day than the normal rabbit.

DISCUSSION

The aim of this work was to investigate in rabbits some of the effects of cholesterol feeding on lipoprotein distribution and metabolism. It was known from several previous reports (3, 4, 9) that cholesterol feeding results in elevated concentration, increased size, and dramatic compositional change in the d < 1.006 g/ml lipoprotein material. Our results further confirmed these effects. We report the new and unexpected finding, however, that virtually all LDL and HDL material can disappear from rabbit plasma as a consequence of cholesterol feeding. LDL and HDL have been isolated previously from cholesterol-fed rabbits by Stange et al. (8) and Pinon and Bridoux (13). It is possible that procedural differences account for this discrepancy between results. Stange et al. (8) used a different type of commercial chow, and studied male rabbits of a different strain as did Pinon and Bridoux (13). It has been well documented that different strains of rabbits can differ in their dietary-induced cholesterolemic response (37). Shore et al. (4) used conditions very similar to ours,

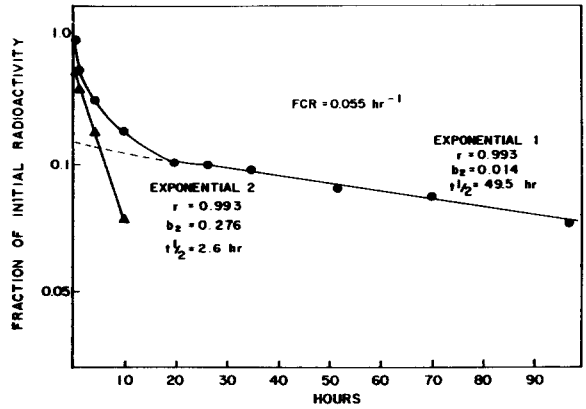

Fig. 13. Decay of plasma ^{125}I-labeled apoE in a cholesterol-fed rabbit; 2.5×10^6 cpm of ^{125}I-labeled apoE in 1.2 ml of the standard buffer was injected.

except for the source of commercial rabbit chow. They also showed that the d < 1.019 g/ml lipoproteins were increased and HDL greatly reduced. However, they found a marginal elevation of LDL (d 1.019–1.063 g/ml). Our rabbits responded to the high dietary cholesterol load with a rapid and sustained alteration in lipoprotein distribution that was fully reversible upon returning the animals to commercial rabbit chow. An interesting observation in our experiments was that cholesterol feeding can cause typical LDL to disappear completely from the circulation. One explanation for this phenomenon, not previously reported, could be that LDL does not disappear but that its typical physical chemical characteristics are altered. According to this view, the dietary cholesterol challenge would cause LDL to become larger and less dense until it could not be distinguished from VLDL on the basis of size or ultracentrifugal flotation behavior. In nonhuman primates, cholesterol feeding can cause the replacement of normal LDL by LDL of increased size and particle weight (38). Alternatively, the failure to detect LDL in our animals could be due to the true absence of both typical and altered LDL. This could be the result of an impaired formation of LDL from its putative precursor, VLDL, which is normally converted to LDL by a mechanism involving stepwise delipidation of the triglyceride-rich lipoproteins by lipoprotein lipase (39). Cholesterol feeding in the rabbit does not alter the clearance rates of triglycerides from the circulation (34, 40). Therefore, the disappearance of LDL could be caused by replacement of normal precursor VLDL by abnormal d < 1.006 g/ml lipoproteins whose catabolism does not lead to formation of IDL and LDL. Camejo et al. (9) showed that the larger VLDL population from cholesterol-fed rabbits was cleared from the circulation of control rabbits very rapidly and that the nonexchangeable cholesteryl esters of these particles were not found later in the LDL or HDL fractions. Kushwaha and Hazzard (10) found that the same was true for the protein moiety of VLDL of the cholesterol-fed rabbit. In the normal rabbit, chylomicron remnants are rapidly removed from the circulation by the liver (41). Using radioactive dietary retinol, Ross and Zilversmit (34) demonstrated that the majority of the d < 1.019 g/ml lipoproteins of cholesterol-fed rabbits are indeed chylomicron remnants. In the cholesterol-fed state, removal of remnants by the liver is impaired (34, 40) because of saturation and reduction in the number of hepatic receptors (42).

Our data show that cholesterol feeding raises the plasma level of apoE by causing an overproduction of this apolipoprotein. Qualitatively similar results have been reported from studies with rats (43). This overproduction could occur in the liver, a major site of apoE synthesis (44), or in macrophages and/or macrophage-derived cells. Monolayers of mouse peritoneal macrophages synthesize and secrete apoE (45). Production of apoE is stimulated 3- to 8-fold in the presence of acetyl-LDL or VLDL from cholesterol-fed rabbits (45). In good agreement with these in vitro results, the in vivo turnover data from our cholesterol-fed rabbit suggest a 4-fold overproduction of the protein.

The reason for the absence of mature spherical HDL in the cholesterol-fed state is not clear. Potential sources for HDL formation are *a*) intravascular lipolysis of triglyceride-rich lipoproteins (46–49) which is not impaired in the cholesterol-fed state (34, 40); and *b*) intestine and liver which secrete nascent discoidal precursor HDL (50, 51). Since we have observed LCAT activity apparently sufficient for formation of spherical HDL, we are led to believe that the discoidal HDL precursors containing both LCAT-activator and LCAT-substrates, i.e., apoA-I, cholesterol, and phosphatidylcholine, must not be formed. Using electron microscopy, Stange et al. (8) were able to find some discoidal HDL upon cholesterol feeding. These particles could correspond to the apoE/phospholipid discs secreted by cholesterol laden-macrophages (52).

To what extent the alteration of lipoprotein distribution is related to the accelerated atherosclerosis in the hypercholesterolemic rabbit[4] has yet to be elucidated. Zilversmit has proposed that cholesterol-enriched remnant particles contribute to atherosclerosis by direct interaction with the arterial wall (53). Macrophages possess a high affinity receptor for chylomicron remnants and can accumulate cholesteryl esters via this route (54). The above hypothesis (53) could be strongly supported by our study which shows that typical LDL, widely implicated as atherogenic particles, can be completely absent in this pathologic condition.

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REFERENCES

1. Roth, R. I., and J. R. Patsch. 1978. Metabolism of the "arginine-rich" protein in the rabbit. *Federation Proc.* **37:** 1322 (Abstract).
2. Duff, G. L. 1935. Experimental cholesterol arteriosclerosis and its relationship to human arteriosclerosis. *Arch. Pathol.* **20:** 82–123, 259–304.

[4] Our hypercholesterolemic rabbits that demonstrated the described lipoprotein pattern developed, within 2–3 months, extensive aortic lesions and corneal arcus.

3. Shumaker, V. N. 1956. Cholesterolemic rabbit lipoproteins—serum lipoproteins of cholesterolemic rabbits. *Am. J. Physiol.* **184:** 35–42.
4. Shore, V. G., B. Shore, and R. G. Hart. 1974. Changes in apolipoproteins and properties of rabbit very low density lipoproteins on induction of cholesteremia. *Biochemistry.* **13:** 1579–1585.
5. Wissler, R. W., and D. Vesselinovitch. 1974. Differences between human and animal atherosclerosis. *In* Atherosclerosis III. G. Schettler and A. Weizel, editors. Springer-Verlag, Berlin, Heidelberg. 319–325.
6. Anitschkow, A. 1933. Experimental arteriosclerosis in animals. *In* Arteriosclerosis. E. V. Cowdry, editor. The Macmillan Company, New York. 271–322.
7. Kritchevsky, D., A. W. Moyer, W. C. Tesar, J. B. Logan, R. A. Brown, M. D. Davies, and H. R. Cox. 1954. Effect of cholesterol vehicle in experimental atherosclerosis. *Am. J. Physiol.* **178:** 30–32.
8. Stange, E., B. Agostini, and J. Papenberg. 1975. Changes in rabbit lipoprotein properties by dietary cholesterol, and saturated and polyunsaturated fats. *Atherosclerosis.* **22:** 125–148.
9. Camejo, G., V. Bosch, C. Arreaza, and H. C. Mendez. 1973. Early changes in plasma lipoprotein structure and biosynthesis in cholesterol-fed rabbits. *J. Lipid Res.* **14:** 61–68.
10. Kushwaha, R. S., and W. R. Hazzard. 1978. Catabolism of very low density lipoproteins in the rabbit. Effect of changing composition and pool size. *Biochim. Biophys. Acta.* **528:** 176–189.
11. Garlick, D. G., F. C. Courtice, and M. Munoz-Marcus. 1965. Plasma lipoproteins in hyperlipaemic states in man and in the rabbit. *Australas. Ann. Med.* **14:** 102–110.
12. Gofman, J. W., F. Lindgren, H. Elliott, W. Mantz, J. Hewitt, B. Striwoser, and V. Herring. 1950. The role of lipids and lipoproteins in atherosclerosis. *Science.* **111:** 166–171.
13. Pinon, J-C., and A. M. Bridoux. 1977. High density lipoproteins in cholesterol-fed rabbits: progressive enrichment with free cholesterol. *Artery.* **3:** 59–71.
14. Patsch, W., J. R. Patsch, G. M. Kostner, S. Sailer, and H. Braunsteiner. 1978. Isolation of subfractions of human very low density lipoproteins by zonal ultracentrifugation. *J. Biol. Chem.* **253:** 4911–4915.
15. Patsch, J. R., S. Sailer, and H. Braunsteiner. 1975. Lipoproteins of the density 1.006–1.020 in the plasma of patients with type III hyperlipoproteinemia in the postabsorptive state. *Eur. J. Clin. Invest.* **5:** 45–55.
16. Patsch, J. R., S. Sailer, G. Kostner, F. Sandhofer, A. Holasek, and H. Braunsteiner. 1974. Separation of the main lipoprotein density classes from human plasma by rate-zonal ultracentrifugation. *J. Lipid Res.* **15:** 356–366.
17. Rudel, L. L., J. A. Lee, M. D. Morris, and J. M. Felts. 1974. Characterization of plasma lipoproteins separated and purified by agarose-column chromatography. *Biochem. J.* **139:** 89–95.
18. Ackers, G. K. 1967. A new calibration procedure for gel filtration columns. *J. Biol. Chem.* **242:** 3237–3238.
19. Patsch, J. R., K. C. Aune, A. M. Gotto, and J. D. Morrisett. 1977. Isolation, chemical characterization, and biophysical properties of three different abnormal lipoproteins: LP-X_1, LP-X_2 and LP-X_3. *J. Biol. Chem.* **252:** 2113–2120.
20. Lees, R. S., and F. T. Hatch. 1963. Sharper separation of lipoprotein species by paper electrophoresis in albumin-containing buffer. *J. Lab. Clin. Med.* **61:** 518–528.
21. Mahley, R. W., and K. H. Weisgraber. 1973. An electrophoretic method for the quantitative isolation of human and swine plasma lipoproteins. *Biochemistry.* **13:** 1964–1969.
22. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
23. Roth, R. I., R. L. Jackson, H. J. Pownall, and A. M. Gotto. 1977. Interaction of plasma "arginine-rich" apolipoprotein with dimyristoylphosphatidylcholine. *Biochemistry.* **16:** 5030–5036.
24. Kane, J. P., T. Sata, R. L. Hamilton, and R. J. Havel. 1975. Apoprotein composition of very low density lipoproteins of human serum. *J. Clin. Invest.* **56:** 1622–1634.
25. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
26. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
27. Manual of Laboratory Methods. Lipid Research Clinics Program, Chapel Hill, NC, University of North Carolina. 1972.
28. Röschlau, P., E. Bernt, and W. Gruber. 1974. Enzymatische Bestimmung des Gesamt-Cholesterins im Serum. *Z. Klin. Chem. Klin. Biochem.* **12:** 403–407.
29. Patsch, W., S. Sailer, and H. Braunsteiner. 1976. An enzymatic method for the determination of the initial rate of cholesterol esterification in human plasma. *J. Lipid Res.* **17:** 182–185.
30. Langer, T., W. Strober, and R. I. Levy. 1972. The metabolism of low density lipoprotein in familial type II hyperlipoproteinemia. *J. Clin. Invest.* **51:** 1528–1536.
31. McFarlane, A. S. 1958. Efficient trace-labeling of proteins with iodine. *Nature.* **182:** 58.
32. Matthews, C. M. E. 1957. The theory of tracer experiments with ^{131}I-labeled plasma proteins. *Phys. Med. Biol.* **2:** 36–53.
33. Laurell, C-B. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* **15:** 45–52.
34. Ross, A. C., and D. B. Zilversmit. 1977. Chylomicron remnant cholesteryl esters as the major constituent of very low density lipoproteins in plasma of cholesterol-fed rabbits. *J. Lipid Res.* **18:** 169–181.
35. Seidel, D. 1979. Electrophoresis of plasma lipoproteins. *In* Report of the HDL Methodology Workshop. K. Lippel, editor. NIH Publication No. 79-1661.
36. Chapman, M. J. 1980. Animal lipoproteins: chemistry, structure, and comparative aspects. *J. Lipid Res.* **21:** 789–853.
37. Roberts, D. C. K., C. E. West, T. G. Redgrave, and J. B. Smith. 1974. Plasma cholesterol concentration in normal and cholesterol-fed rabbits. *Atherosclerosis.* **19:** 369–380.
38. Rudel, L. L., L. L. Pitts, and C. A. Nelson. 1977. Characterization of plasma low density lipoproteins of non-human primates fed dietary cholesterol. *J. Lipid Res.* **18:** 211–222.
39. Behr, S. R., J. R. Patsch, T. Forte, and A. Bensadoun. 1981. Plasma lipoprotein changes resulting from immunologically blocked lipolysis. *J. Lipid Res.* **22:** 443–451.
40. Redgrave, T. G., K. B. Dunne, D. C. K. Roberts, and C. E. West. 1976. Chylomicron metabolism in rabbits fed diets with or without added cholesterol. *Atherosclerosis.* **24:** 501–508.
41. Redgrave, T. G. 1970. Formation of cholesteryl ester-rich

particulate lipid during metabolism of chylomicrons. *J. Clin. Invest.* **49:** 465–471.
42. Kovanen, P. T., M. S. Brown, S. K. Basu, D. W. Bilheimer, and J. L. Goldstein. 1981. Saturation and suppression of hepatic lipoprotein receptors: a mechanism for the hypercholesterolemia of cholesterol-fed rabbits. *Proc. Natl. Acad. Sci. USA.* **78:** 1396–1400.
43. Wong, L., and D. Rubinstein. 1979. Turnover of apoE in normal and hypercholesterolemic rats. *Atherosclerosis.* **34:** 249–258.
44. Felker, T. E., M. Fainaru, R. L. Hamilton, and R. J. Havel. 1977. Secretion of the arginine-rich and A-I apolipoproteins by the isolated perfused rat liver. *J. Lipid Res.* **18:** 465–473.
45. Basu, S. K., M. S. Brown, Y. K. Ho, R. J. Havel, and J. L. Goldstein. 1981. Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E. *Proc. Natl. Acad. Sci. USA.* **78:** 7545–7549.
46. Chajek, T., and S. Eisenberg. 1978. Very low density lipoprotein. Metabolism of phospholipids, cholesterol, and apolipoprotein C in the isolated perfused rat liver. *J. Clin. Invest.* **62:** 1654–1665.
47. Patsch, J. R., A. M. Gotto, S. Eisenberg, and T. Olivecrona. 1978. Formation of high density lipoprotein$_2$-like particles during lipolysis of very low density lipoproteins in vitro. *Proc. Natl. Acad. Sci. USA.* **75:** 4519–4523.
48. Redgrave, T. G., and D. M. Small. 1979. Quantitation of the transfer of surface phospholipid of chylomicrons of the high density lipoprotein fraction during the catabolism of chylomicrons in the rat. *J. Clin. Invest.* **64:** 162–171.
49. Tall, A. R., P. M. R. Green, R. M. Glickman, and J. W. Riley. 1979. Metabolic fate of chylomicron phospholipids and apoproteins in the rat. *J. Clin. Invest.* **64:** 977–989.
50. Hamilton, R. L., M. C. Williams, C. J. Fielding, and R. J. Havel. 1976. Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. *J. Clin. Invest.* **58:** 667–680.
51. Green, P. H. R., A. R. Tall, and R. M. Glickman. 1978. Rat intestine secretes discoidal high density lipoprotein. *J. Clin. Invest.* **61:** 528–534.
52. Basu, S. K., Y. K. Ho, M. S. Brown, D. W. Bilheimer, R. G. W. Anderson, and J. L. Goldstein. 1982. Biochemical and genetic studies of the apoprotein E secreted by mouse macrophages and human monocytes. *J. Biol. Chem.* **257:** 9788–9795.
53. Zilversmit, D. B. 1979. Atherogenesis: a postprandial phenomenon. *Circulation.* **60:** 473–485.
54. Goldstein, J. L., Y. K. Ho, M. S. Brown, T. L. Innerarity, and R. W. Mahley. 1980. Cholesteryl ester accumulation in macrophages resulting from receptor-mediated uptake and degradation of hypercholesterolemic canine β-very low density lipoproteins. *J. Biol. Chem.* **255:** 1839–1848.