Effects of high cholesterol diets on rat plasma lipoproteins and lipoprotein-cell interactions

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Abstract High fat, high cholesterol diets do not produce atherosclerotic lesions in some animal species such as the rat; however, when combined with experimentally induced hypothyroidism, such diets do produce lesions. While the diets or hypothyroidism each induce significant alterations in plasma lipoproteins, the combination produces marked hypercholesterolemia. If the atherosclerosis is related to the hyperlipidemia, the combination regimen could be provoking changes in the structure or compositions of lipoproteins which are not noted with either regimen alone. To test this hypothesis, Sprague-Dawley male rats (~250 g) were treated as follows: Diet(a) = chow + 5% lard and 0.3% Na taurocholate; Diet(b) = Diet(a) + 2% cholesterol; Diet(c) = Diet(b) + 0.1% propylthiouracil (PTU). The major findings were as follows. *1*) With Diet(b), slow floating very low density lipoprotein (VLDL) (pre-β) enriched in cholesteryl esters accumulated in plasma and low density lipoprotein (LDL) disappeared from its usual flotation position. *2*) With Diet(c), changes in plasma concentration were more marked but were also qualitatively different. More VLDL accumulated, and distribution of VLDL was shifted toward even slower floating cholesteryl ester-rich particles. VLDL had "broad β" mobility. Also, a β-migrating intermediate density lipoprotein (IDL) population appeared. *3*) Lipoprotein ($d < 1.019$ g/ml) and zonal subfractions of $d < 1.019$ g/ml lipoproteins (isolated from rats on cholesterol Diet(b)) stimulated [^{3}H]oleate incorporation into cholesteryl esters of fibroblasts and macrophages, while the $d < 1.019$ g/ml fractions of 5% fat (Diet(a))-fed rats did not. *4*) The major finding of this study was that identically prepared $d < 1.019$ g/ml fractions of Chol + PTU-treated rats (Diet(c)) were ~2.5-fold more stimulatory than the lipoproteins of cholesterol-fed rats. The results could not be explained by differences in cholesterol contents of the cholesterol-rich lipoproteins, but significant differences in the apoprotein compositions of the fraction were found which could be important. The most active fractions had higher $apoB_L/apoB_S$ and apoE/apoC ratios than less active fractions. Thus, the combination regimen of cholesterol and PTU produced changes in lipoprotein structure and composition which enhanced the abilities of the lipoproteins to interact with cells. The results suggest that analysis of lipoprotein-cell interactions in vitro may be predictive of the atherogenic potential of lipoproteins in vivo and that euthyroidism in rat protects against atherogenic hyperlipidemia.—**Cole, T. G., I. Kuisk, W. Patsch, and G. Schonfeld.** Effects of high cholesterol diets on rat plasma lipoproteins and lipoprotein-cell interactions. *J. Lipid Res.* 1984. **25:** 593–603.

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Significant changes are produced in plasma lipoproteins by high fat, high cholesterol diets in man (1–8), and in several other species including nonhuman primates (9–13), swine (14, 15), guinea pigs (16), dogs (17), rabbits (18, 19), and rats (20, 21). Although there are considerable differences between the responses of different species, in general, total cholesterol levels rise and there is a tendency for β-migrating VLDL and IDL to accumulate in plasma (22–24). LDL is variably affected; for example, it increases in man (1, 2), but virtually disappears from its usual flotation position upon zonal ultracentrifugation of rabbit plasma (19). Another class of lipoproteins designated as HDL_C or HDL_1 also accumulates in many species. The appearance in plasma of β-VLDL and/or IDL is of great interest because their presence is correlated with the development of atherosclerotic lesions (9, 11, 21, 22). In addition, these lipoproteins interact avidly with macrophages (25, 26) and arterial smooth muscle cells (27, 28), structures important in atherogenesis (29, 30), whereas the α-migrating VLDL and LDL present in animals on normal diets interact less well. Dietary cholesterol-induced β-VLDL and IDL are therefore thought to play important roles in atherogenesis.

It has been known for some time that diet-induced hyperlipidemia and atherosclerotic lesions develop more easily in some species than others. For example, rabbits and swine develop hyperlipidemia and arterial lesions on less extensively modified diets than do dogs and rats (31). Indeed, for the latter species to develop lesions, the diets have to include not only large amounts of fat and cholesterol, but also bile acids, and, in addition, the animals usually are rendered hypothyroid or deficient in essential fatty acids (17, 21, 31). Recently, it was demonstrated in

Abbreviations: VLDL, IDL, LDL, and HDL are very low, intermediate, low, and high density lipoproteins, respectively; SDS, sodium dodecyl sulfate; PTU, propylthiouracil; $apoB_L$, large apoB, also known as apoB-100; $apoB_S$, small apoB, also known as apoB-48; TG, triglyceride; ACAT, acyl CoA:cholesterol acyltransferase; LPDS, lipoprotein-deficient serum; NBCS, newborn calf serum; MEM, Minimum Essential Medium; DME, Dulbecco's modified Eagle's medium; RIA, radioimmunoassay.

the rat that hypothyroidism alone produces certain alterations in plasma lipoprotein and apoprotein levels and lipoprotein metabolism (32–36), and that the alterations produced by hypothyroidism plus a high fat, high cholesterol diet are quite different from those produced by the diet or by hypothyroidism alone (37). If qualitative and quantitative changes in plasma lipoproteins are related to atherosclerosis, and if lipoprotein-cell interactions do indeed reflect the potential of lipoproteins to play a role in atherogenesis, the lipoproteins present in plasma on more effective atherogenic treatments (21, 38–40) ought to interact more avidly with cells than the lipoproteins present under less atherogenic conditions. The aim of the present work was to describe, in the rat, diet- and hypothyroidism-induced changes in plasma lipoproteins, and to test the interactions of lipoproteins with cultured cells.

MATERIALS AND METHODS

Animals and diets

Male Sprague-Dawley rats weighing 265 ± 17 g (mean ± SD) were used. Diets were fed ad libitum for 19–21 days. Experimental diets were fed as indicated in **Table 1:** Diet(a): Wayne Lab Blox rat chow (Allied Mills, Inc., Chicago, IL) containing 24.5% (w/w) protein, 4.3% fat, 7.8% ash, 13.3% H_2O, 3.7% fiber, and 47.4% nitrogen-free carbohydrate of which <5% was mono- or disaccharides plus 5% lard and 0.3% Na taurocholate; Diet(b): Diet(a) plus 2% cholesterol; Diet(c): Diet(b) plus 0.1% PTU. Diets(b) and (c) were prepared by ICN Nutrition Biochemicals. Weight gain was similar on Diets(a) and (b) ranging from 32 to 39 g per week per group. On the PTU-containing diet, weight gains ranged from 13 to 19 g per week per group (32, 33).

Animals were fasted from 0900 to 1230, anesthetized with diethylether, and exsanguinated via their inferior venae cava. Blood was collected on ice in tubes containing 1 mg/ml EDTA, and plasmas were separated by centrifugation. Seven plasma pools for each dietary group were prepared, each pool consisting of four to ten individual rat plasma samples.

Lipoprotein characterizations

Lipoprotein distributions were assessed by molecular sieving chromatography and by ultracentrifugation in fixed-angle and zonal rotors. For the former, 2-ml plasma samples were loaded on a 90 × 1.5 cm column containing Sepharose 6B (Pharmacia, Piscataway, NJ), equilibrated with 1 mM EDTA, 0.15 M saline, 0.01% Na azide, pH 8.2. Plasma pools were adjusted to density 1.019 or 1.070 g/ml, as indicated, by the addition of solid KBr and ultracentrifuged for 1.8×10^8 g-min (50.2 rotor, Beckman Instruments, Palo Alto, CA, 20 hr, 40,000 rpm, 4°C). Infranates from the d 1.070 g/ml runs were adjusted to d 1.21 g/ml with solid KBr and spun for 2.5×10^8 g-min (50.2 Ti rotor, 24 hr, 50,000 rpm, 4°C). In some cases, infranates of d 1.019 g/ml runs were centrifuged at d 1.070 g/ml and again at d 1.21 g/ml. Each fraction was washed by ultracentrifugation at its higher density limit. Aliquots of d < 1.019, d < 1.070, and d > 1.070 g/ml fractions, prepared by single spins in the 50.2 rotor, were subjected to zonal ultracentrifugation. D < 1.070 and d < 1.019 g/ml fractions were analyzed in a linear NaBr density gradient (1.00–1.15 g/ml) at 42,000 rpm, 45 min, 15°C (41) and also in a linear gradient spanning the density of 1.00 to 1.30 g/ml at 42,000 rpm, 140 min, 15°C (42). D > 1.070 g/ml fractions were analyzed in a nonlinear three-step NaBr gradient in the density range of 1.00 to 1.30 g/ml for 18 hr at 41,000 rpm and 15°C (43). Ti-14 rotors were used for all zonal runs.

Triglyceride (TG) and cholesterol in plasma were determined by standard procedures with coefficients of variation of 3–5% for quality control samples (44). Lipoprotein electrophoresis was performed on 20-μl aliquots of plasma pools or lipoprotein fractions according to Noble (45). For analyses of compositions, lipoprotein fractions were dialyzed against EDTA-saline and analyzed for free and esterified cholesterol and TG (Boehringer

TABLE 1. Diet-induced changes in rat plasma lipids and apoproteins

Diets	TG	Chol	ApoA-I	ApoB	ApoE
			mg/dl		
(a) Fat	72 ± 27	62 ± 4	48 ± 6	13 ± 1	24 ± 8
(b) Chol	62 ± 17	112 ± 29[a,c]	37 ± 8[a,c]	13 ± 1 (19 ± 5)[a,c]	13 ± 3[a,c]
(c) Chol + PTU	29 ± 5[a,b]	251 ± 43[a,b]	45 ± 3	34 ± 2[a,b]	32 ± 7[b,c]

Means ± 1 SD are given. Lipids were obtained on seven plasma pools for each diet, each pool was from four to ten rats. Apoproteins were obtained by RIA on six plasma pools per diet, each pool from four rats. Superscripts *a*, *b*, *c*, denote those dietary groups that are significantly different from the means bearing the superscripts ($P < 0.05$). ApoB results in parentheses were obtained by TMU precipitation of d < 1.070 g/ml fractions (see Table 2).

Enzymatic Kits, Indianapolis, IN), and phospholipid (PL) (46). Protein was determined according to the method of Lowry et al. (47) as modified by Bensadoun and Weinstein (48) using bovine serum alumin as standard. Levels of apoproteins B, A-I, and E were determined by radioimmunoassays (49–51) in plasma or column effluents. Lipoprotein fractions were also assessed for their apoprotein compositions by sodium dodecyl sulfate electrophoresis in gradient gels (3.5%–20% polyacrylamide) (52, 53), or by isoelectric focusing (54). After staining and destaining, gels were scanned with a laser densitometer (Zeineh Soft Laser Scanning Densitometer, Biomed Instruments, Inc., Chicago, IL) and scans were integrated with a Hewlett Packard 9864 digitizer (55). Bands were identified by their apparent molecular weights using protein standards run simultaneously. Linearity between load of protein and dye area uptake was established for loads between 10 and 40 μg of lipoprotein-protein. Loads of 5 or 60 μg departed from linearity.

Interactions of lipoproteins with cells

Fibroblasts were cultured from skin explants taken from a young Sprague-Dawley rat (56). Cells were passaged weekly and used for experiments between the fifth and fifteenth passage. On day 1 of an experiment, cells were plated at a density of 10^4 cells/cm^2 in 35-mm dishes in 1.5 ml of Minimum Essential Medium (MEM, Gibco, Grand Island, NY) containing 15% newborn calf serum (NBCS), fungizone (0.63 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). On day 5, cells were washed with saline G, and 1.5 ml of MEM, containing 10% lipoprotein-deficient serum (LPDS) was added. On day 7, lipoprotein interaction studies were performed using lipoproteins that had been stored under sterile conditions at 4°C for less than 1 week.

Rat peritoneal macrophages were harvested from nonstimulated male Sprague-Dawley rats by lavage of the peritoneal cavity with phosphate-buffered saline containing 110 mg/dl glucose and heparin (5 U/ml) (57). Cells were sedimented by centrifugation and washed twice before seeding in Dulbecco's modified Eagle's Medium (DME, Gibco) containing 20% NBCS at a density of 4 $\times$ 10^4 cells/cm^2 in 35-mm dishes. Nonadherent cells were removed after 2 hr, and macrophages were incubated for 18 hr before lipoprotein interaction studies. Rat rather than mouse cells were used in order to avoid crossing of species.

Interactions of lipoproteins with rat fibroblasts and macrophages were assessed by measuring acyl CoA:cholesterol acyltransferase (ACAT) activity as a function of medium lipoprotein concentration (58). Increasing concentrations of lipoproteins were added to cells in 0.7 ml of MEM-LPDS (fibroblasts) or DME-LPDS (macrophages). After an incubation period of 5 hr at 37°C, 0.1 ml of MEM or DME containing [^{3}H]oleic acid (New England Nuclear, Boston, MA, 86 Ci/mole) complexed to bovine serum albumin (Fraction V, Sigma-Aldrich, St. Louis, MO) was added to give a final concentration of 0.14 mM oleic acid. Specific activity of oleic acid was 30.4 cpm/pmol in the fibroblast experiments and 18 cpm/pmol in the macrophage experiments, respectively. Following an incubation period of 18 hr at 37°C, dishes were placed on ice, media were removed, and cells were washed five times with saline. Cells were harvested by using a rubber policeman and cellular lipid was extracted (59). Cholesteryl esters were separated by thin-layer chromatography and radioactivity was determined. Incorporation of [^{3}H]oleic acid into cholesteryl esters is given in pmol per mg cell protein during the 18-hr incubation period.

Calculations

All variables were examined by univariate analysis, log-transformed if kurtosis exceeded 2.0 or if skewness exceeded 1.5, and statistical significance at $P \leq 0.05$ was determined by analysis of variance. Unpaired *t*-tests also were performed (60).

RESULTS

On Diet(b), plasma cholesterol and apoB levels increased and levels of apoE decreased (Table 1 and **Table 2**). Diet(c) increased plasma cholesterol, apoB, and apoE levels, while triglycerides were lower. Since the radioimmunoassay of apoB utilized LDL as standard which contains between 90 and 95% of the larger form of apoB (B_L) (50, 61), the assay could be subject to error in quantifying total plasma apoB values. Therefore, apoB levels also were obtained by two other methods (Table 2). In general, the methods yielded results which were in good agreement for the fat and the Chol + PTU groups. However, apoB levels in the Chol group may have been underestimated by RIA. This may be explained by differences in the immunoreactivity of apoB in relatively large VLDL particles and/or the prominence of the small apoB

TABLE 2. Plasma apoB levels

Diets	SDS-Gels[x]	TMU[y]	RIA[z]
		mg/dl	
(a) Fat	12 ± 4	10 ± 2	13 ± 1
(b) 2% Chol	20 ± 5	19 ± 5	13 ± 1
(c) 2% Chol + PTU	30 ± 4	32 ± 5	34 ± 2

[x] Calculated from dye uptake on polyacrylamide 3.5–20% gels and amount of protein in d < 1.07 g/ml fraction.
[y] Determined in d < 1.07 g/ml fractions as protein insoluble in 4.2 M tetramethylurea (TMU).
[z] Determined in plasma by radioimmunoassay.

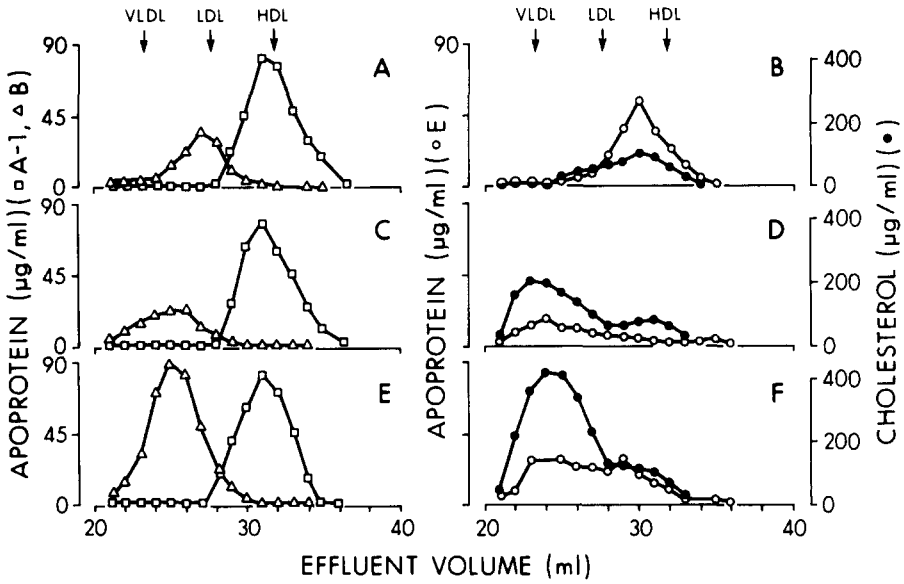

Fig. 1. Gel permeation chromatographic profiles of plasma cholesterol and apoA-I, apoB, and apoE. Two ml of pooled plasma was filtered. Cholesterol was determined enzymatically; apoproteins were determined by radioimmunoassay. Recoveries of cholesterol and apoproteins from the column ranged from 83 to 97%. A and B, fat diet (a); C and D, cholesterol diet (b); E and F, cholesterol + PTU diet (c).

subspecies, $apoB_S$, in these VLDL (see Table 4). $ApoB_S$ is less reactive in our assays than is $apoB_L$ (61).

The distribution of apoproteins among the different lipoproteins was determined by molecular sieving chromatography (**Fig. 1**). On Diet(a), apoB was found mainly in the LDL region, and apoA-I and most of the cholesterol eluted in the HDL region. This distribution is comparable with the elution of the major classes of rat lipoproteins isolated from plasma by preparative ultracentrifugations (see elution positions indicated by arrows). ApoE eluted between apoB and apoA-I where HDL_1 or HDL_C are expected to elute (62). Following Chol feeding (Diet b), the proportion of cholesterol increased in the VLDL elution volume and even exceeded the amount of cholesterol present in HDL. Concomitantly, while the distribution of apoA-I was not greatly changed, both apoB and apoE were shifted toward the VLDL region. These changes are compatible with the appearance in plasma of large cholesterol- and apoE-rich particles and decreases in HDL_1 or HDL_C of the usual size. Even greater redistribution of cholesterol and apoB into the VLDL region was observed in the plasma of Chol + PTU-treated (Diet c) animals. ApoE now was present in both the VLDL and the HDL elution volumes, with the larger fraction being present in the VLDL region. Thus, the combination regimen appeared to further enhance levels of large particles, while also maintaining closer to normal levels of HDL_C of the usual size.

Results compatible with those found on gel permeation chromatography were found on lipoprotein electrophoresis of plasma in agarose, another nonperturbing but also nonquantitative procedure, and on preparative and zonal ultracentrifugations which, although subject to quantitation, may perturb lipoprotein structure (21). On electrophoresis, dietary cholesterol increased the amount of pre-β-migrating lipoproteins while β-lipoproteins disappeared (results not shown). The Chol + PTU regimen produced large amounts of "broad β"-migrating lipoproteins. On preparative ultracentrifugation, the total amounts of d < 1.019 g/ml lipoproteins were nearly trebled by the cholesterol diet and quintupled by the cholesterol + PTU regimen (**Table 3,** last column on

TABLE 3. Compositions and levels of d < 1.019 g/ml lipoproteins

Diets	Protein	FC	CE	TG	PL	Lipoprotein Mass
			% mass			*mg/dl*
(a) Fat	13 ± 1	4 ± 1	10 ± 2	56 ± 4	17 ± 1	57 ± 13
(b) Chol	14 ± 1	5 ± 1	49 ± $3^{a,c}$	15 ± $4^{a,c}$	17 ± 1	146 ± $3^{a,c}$
(c) Chol + PTU	13 ± 2	9 ± $1^{a,b}$	58 ± $2^{a,b}$	3 ± $1^{a,b}$	16 ± 1	285 ± $120^{a,b}$

Results are means ± 1 SD of four d < 1.019 g/ml preparations in each diet group; each fraction was isolated from a plasma pool containing four to ten rats. Superscripts *a*, *b*, *c*, denote those dietary groups that are significantly different from the means bearing the superscripts ($P < 0.05$).

right). Gross diet-induced alterations were seen in the compositions of d < 1.019 fractions (Table 3), i.e., the particles changed from TG-rich to cholesterol-rich lipoproteins. Similar compositional changes were noted in d < 1.070 g/ml fractions (data not shown). No significant concomitant changes were noted in the concentrations of the mass of 1.070–1.21 g/ml lipoproteins (mean ± SD concentrations on the three diets were 89 ± 5, 90 ± 8, 84 ± 21 mg/dl) or in HDL composition (data not shown).

On zonal ultracentrifugation in 1.00–1.30 g/ml gradients, the d < 1.070 g/ml lipoproteins isolated from fat-fed animals floated in two relatively homogeneous populations (**Fig. 2A**) corresponding to VLDL (<70 ml) and LDL (<220 ml) (55, 63). Analogous density fractions isolated from animals fed the Chol and Chol + PTU diets floated as more heterogeneous particles with distinguishable more slowly floating shoulders (~70 ml, Fig. 2A) or second peaks (~100 ml, Fig. 2B). The peak corresponding to LDL was not seen in the d < 1.070 g/ml fractions of either group b or c.

When the same d < 1.070 g/ml fractions were centrifuged in 1.00–1.15 g/ml gradients, the subpopulations became better defined (**Fig. 3**). On each of the three diets two populations were seen, one floating at <80 ml, and a second which was present in increasing quantities and floated progressively more slowly with peaks at 140, 190, and 300 ml on fat, Chol, and Chol + PTU diets, respectively (Fig. 3A). To assure ourselves that the two d

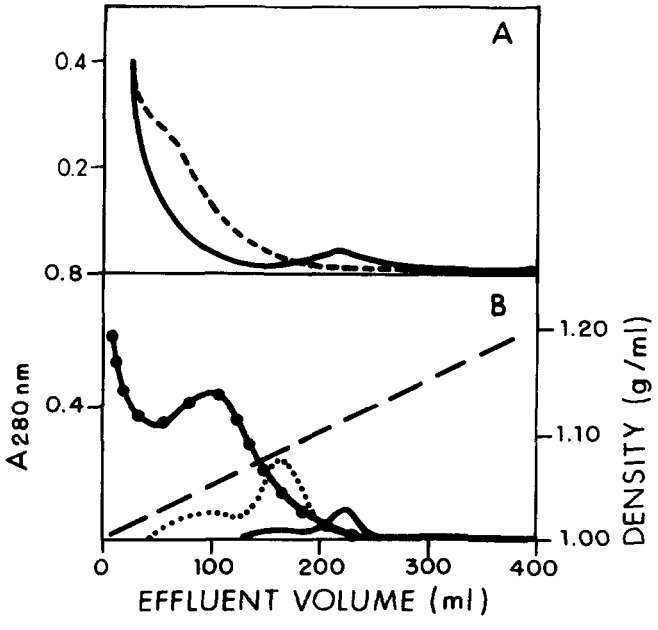

Fig. 2. Zonal ultracentrifugation of rat plasma fractions isolated from 20 ml of pooled plasma. Linear gradient 1.00–1.30 g/ml, at 42,000 rpm, 140 min, 15°C. Panel A, d < 1.070 g/ml: Diet(a) ——; Diet(b) – – –. Panel B, d < 1.070 g/ml: Diet(c) ●—●—●—; d 1.019–1.070 g/ml: Diet(c) · · · · ·; Diet(a) ——.

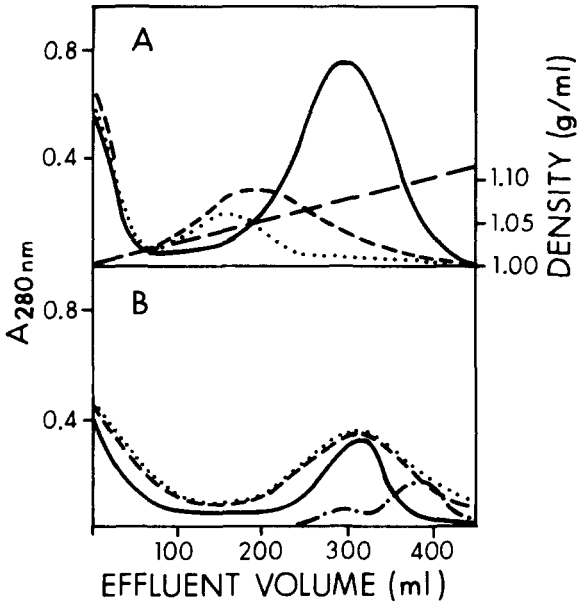

Fig. 3. Zonal ultracentrifugation of rat plasma fractions isolated from 20 ml of pooled plasma. Linear gradient 1.00–1.15 g/ml, 42,000 rpm, 45 min, 15°C. Panel A, d < 1.070 g/ml: Diet(a) · · · · ·; Diet(b) – – –; Diet(c) ——. Panel B, Diet (c): whole plasma · · · · ·; d < 1.070 g/ml – – –; d < 1.019 g/ml ——; d 1.019–1.070 g/ml –·–·.

< 1.070 g/ml populations were not artefacts produced during prior ultracentrifugation in the fixed angle rotors, whole plasma of Chol + PTU-fed rats was subjected to zonal ultracentrifugation in the 1.00–1.15 g/ml gradient. Superimposible effluent profiles were obtained (Fig. 3B).

Since the peak corresponding to LDL was not present in either Chol-fed group in the 1.00–1.30 g/ml gradients, we wished to assess whether any materials were present in the d 1.019–1.070 g/ml range. Therefore, d 1.019–1.070 g/ml density fractions were isolated from plasmas of fat-fed and Chol + PTU-treated animals and ultracentrifuged in the 1.00–1.30 g/ml gradients (Fig. 2B). The d 1.019–1.070 g/ml fraction of fat-fed rats floated primarily in the position of LDL (~220 ml), whereas the analogous fraction of Chol + PTU-treated rats floated at ~160 ml, corresponding to the tail of the slower peak of the d < 1.070 g/ml fraction. Therefore, a "true" LDL indeed was not present in the d < 1.070 g/ml fraction of Chol + PTU-treated rats. Fractions of d < 1.019 and d 1.019–1.070 g/ml of Chol + PTU rats were analyzed also on 1.00–1.15 g/ml gradients (Fig. 3B). The d < 1.019 g/ml fraction floated with a peak of the same position as the whole d < 1.070 g/ml fraction (~300 ml), whereas the d 1.019–1.070 g/ml fraction floated in the tailing region of the d < 1.070 g/ml fraction at ~375 ml. The S_f rate of this slow subpopulation calculated from the rotor calibration curve (41) was ~30 $S^0_{f(1.063)}$, corresponding to IDL. Thus, the broad peak (210–400 ml) formed by plasma or the d < 1.070 g/ml fractions appeared to be comprised of slow floating VLDL and of

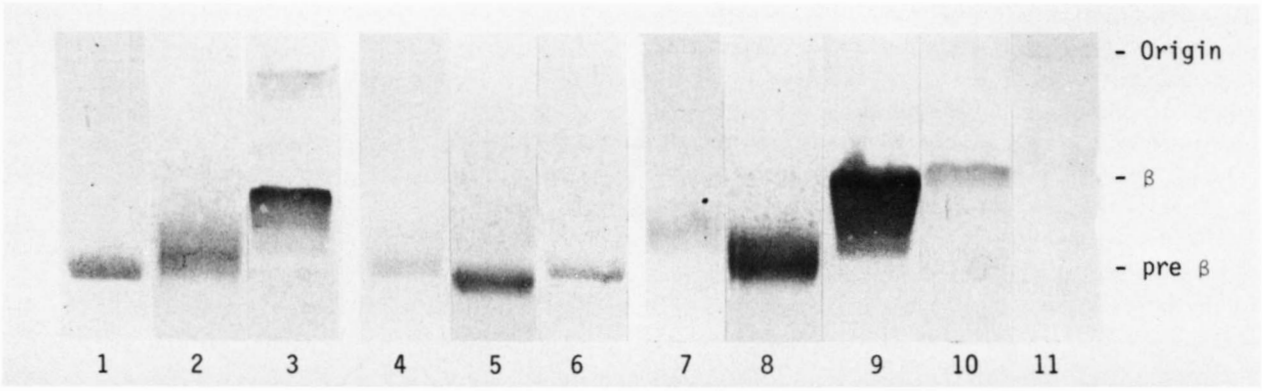

Fig. 4. Lipoprotein electrophoresis of rat lipoprotein fractions isolated by fixed angle rotor and zonal ultracentrifugation. Lanes 1, 4, 7, and 11 are d < 1.019 g/ml, 70–200 ml, 210–400 ml, and LDL fractions of fat-fed rats, respectively. Lanes 2, 5, and 8 are d < 1.019 g/ml, 70–200 ml, and 210–400 ml fractions of Chol-fed rats, respectively. Lanes 3, 6, and 9 are d < 1.019 g/ml, 70–200 ml, and 210–400 ml subfractions of Chol + PTU-fed rats, respectively. Lane 10 is the 1.019–1.070 g/ml fraction of Chol + PTU-fed rats (see Fig. 3 for zonal effluent fractions).

IDL, but the d < 1.019 g/ml fraction contained primarily VLDL.

The various ultracentrifugal fractions were dialyzed against 1 mM EDTA, 0.16 M NaCl, pH 8.0, concentrated by dry dialysis against high molecular weight dextran, and subjected to electrophoresis in 1% agarose (**Fig. 4**). The d < 1.019 g/ml fractions (fixed angle rotor) of fat-fed and Chol-fed rats migrated in the pre-β position, while the d < 1.019 g/ml fractions of Chol + PTU-fed animals migrated as a broad band overlapping the β and pre-β positions. The faster floating zonal subfractions of the d < 1.019 g/ml lipoprotein (70–200 ml effluent volumes, Fig. 3) migrated in the pre-β region for the three diet groups. The slower floating 210–400 ml fractions of fat-fed and Chol-fed rats also migrated as pre-β, while the same zonal fractions of Chol + PTU-fed rats migrated as a broad band primarily in the β position. The d 1.019–1.070 g/ml IDL fraction of Chol + PTU-fed rats (~375 ml, Fig. 3B) migrated as a β lipoprotein.

Zonal elution profiles of the d > 1.070 g/ml fractions of the various animal groups all were qualitatively similar, i.e., only one HDL population was seen corresponding to HDL_2 (55, 64). Neither HDL_1 nor HDL_C was resolved by these runs (data not shown).

Lipoprotein interactions with cells

Since the major diet-induced changes were found in the d < 1.019 g/ml lipoproteins, these fractions and their zonal subfractions (70–200 ml and 210–400 ml) were used for cell interaction studies (**Fig. 5**). In macrophages

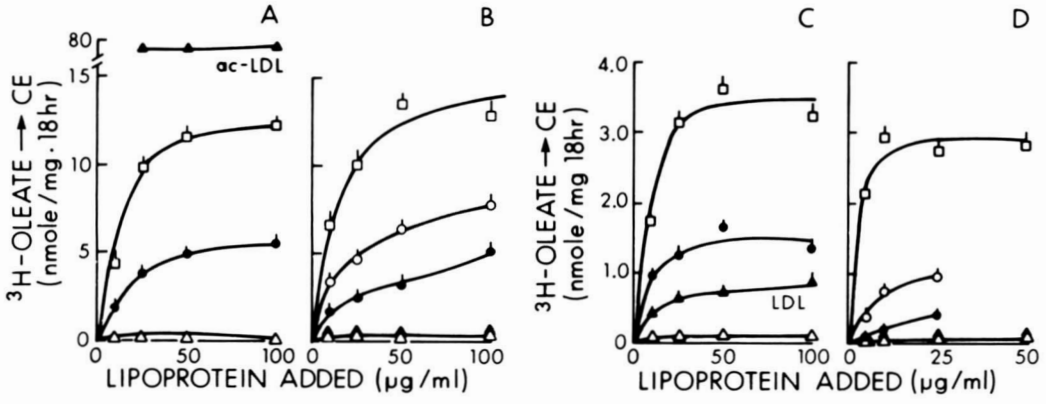

Fig. 5. Lipoprotein-dependent stimulation of [^{3}H]oleate incorporation into cholesteryl esters of cultured rat macrophages (A, B) and cultured rat fibroblasts (C, D). Panel A, stimulation in macrophages by d < 1.019 g/ml fractions of fat-fed (△), Chol-fed (●), and Chol + PTU-fed (□) rats. Stimulation by acetyl LDL (human) also is shown (▲). Panel B, stimulation by 70–200 ml zonal fractions of fat-fed (▲) and Chol-fed (●) rats, and by 210–400 ml zonal fractions of fat-fed (△), Chol-fed (○), and Chol + PTU-fed (□) rats. Panel C, stimulation in fibroblasts by lipoproteins as in Panel A. Stimulation by LDL (human) is shown as (▲). Panel D, stimulation in fibroblasts, lipoproteins as in Panel B. Esterification rates in the absence of lipoproteins were 1.77 ± 0.16 and 0.55 ± 0.04 (mean ± SD, n = three dishes per point) for macrophages and fibroblasts, respectively. These values have been subtracted. Abcissa is in terms of lipoprotein protein. Bars above data points represent one standard deviation.

a lipoprotein dose-dependent stimulation of [^{3}H]oleate incorporation into cholesteryl esters was observed following the addition of the d < 1.019 g/ml fraction isolated from Chol-fed rats. Stimulation of [^{3}H]oleate incorporation was even more pronounced by addition of the same density fraction isolated from PTU + Chol-fed rats, but the d < 1.019 g/ml fraction isolated from fat-fed rats produced very little if any detectable stimulation (Fig. 5A). When the doses of added lipoproteins are expressed on the basis of lipoprotein cholesterol rather than lipoprotein-protein, the d < 1.019 g/ml fraction of Chol + PTU-fed rats still exhibited ~2.5-fold excess maximum stimulating activity. The 210–400 ml fractions were more stimulating than the 70–200 ml fractions (Fig. 5B). As expected (57), the control acetylated human LDL exhibited considerable stimulating activity in macrophages. Studies carried out with d < 1.070 g/ml fractions on another occasion yielded similar results (not shown).

In fibroblasts, stimulation of [^{3}H]oleate incorporation into cholesteryl esters also was much more affected by the d < 1.019 g/ml fractions isolated from Chol + PTU-fed than from Chol-fed rats (Fig. 5C and D) regardless of whether doses of added lipoproteins are expressed in terms of their protein or cholesterol contents. Here too, the 210–400 ml fractions were more effective stimulators. The d < 1.019 g/ml fractions of fat-fed rats exhibited little if any stimulating activity. LDL isolated from chow-fed rats by zonal ultracentrifugation did stimulate in a dose-dependent fashion, but was less effective than any d < 1.019 g/ml fraction. Studies carried out on two other occasions using d < 1.019 and d < 1.070 g/ml lipoproteins yielded compatible results (not shown).

Apoproteins of d < 1.019 g/ml fractions

To this point it has been shown by a variety of techniques that cholesterol and cholesterol + PTU regimens produced increases of VLDL-sized lipoproteins which floated at d 1.019 g/ml in fixed-angle rotors and formed prominent, relatively slow floating lipoproteins in zonal rotors. These lipoproteins were enriched with cholesterol

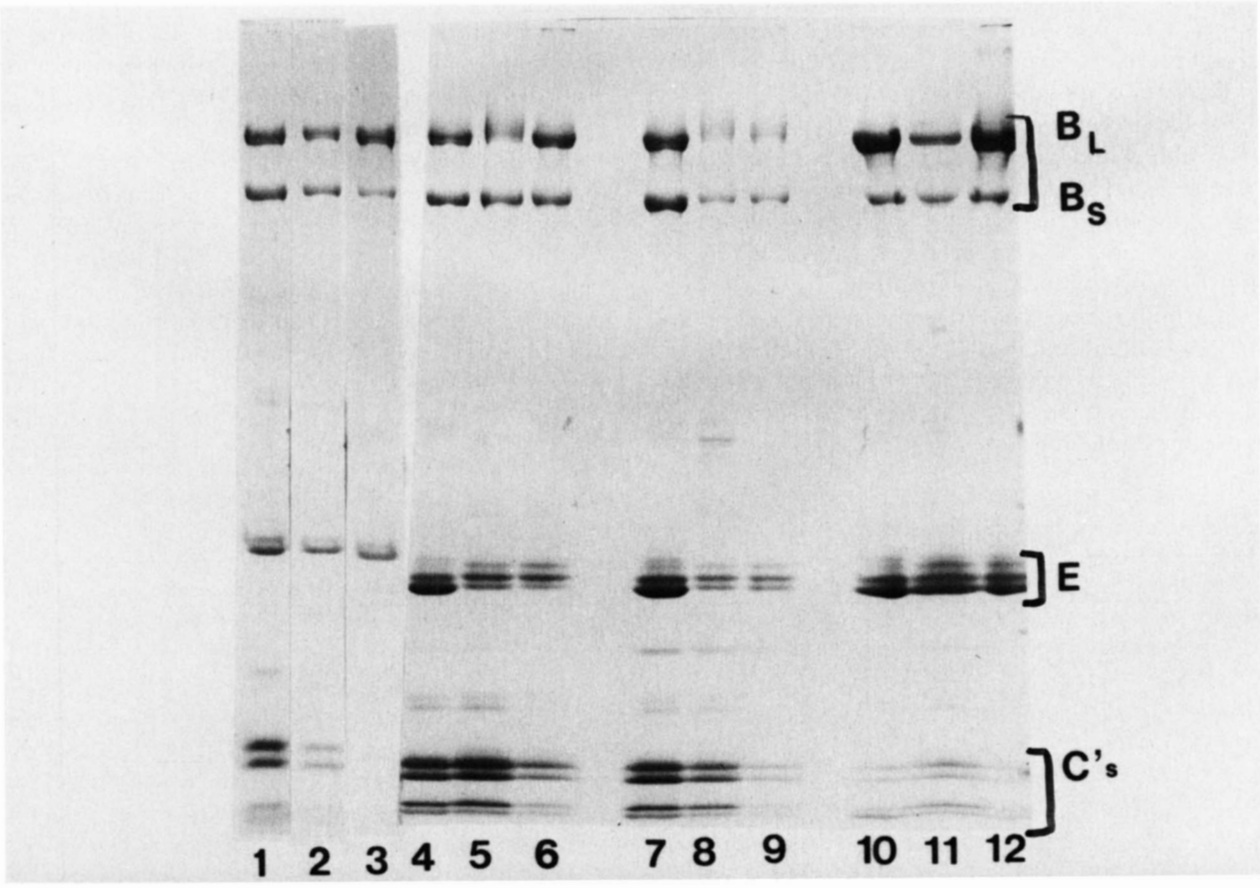

Fig. 6. Gradient (3.5–20%) polyacrylamide gel electrophoresis of rat plasma lipoprotein fractions. Lanes 1, 2, and 3 are d < 1.019 g/ml fractions of fat-fed, Chol-fed, and Chol + PTU-fed rats, respectively. Lanes 4, 5, and 6 are d < 1.019 g/ml, 70–200 ml, and 210–400 ml fractions of fat-fed rats, respectively. Lanes 7, 8, and 9 are respective fractions of Chol-fed rats. Lanes 10, 11, and 12 are from Chol + PTU-fed rats (see Tables 5 and 6 for quantitation).

TABLE 4. Diet effects on apoprotein B_L/B_S ratios of d < 1.019 g/ml lipoproteins

	Fixed Angle Rotor Fractions		Zonal Rotor Fractions	
Dietary Groups	d < 1.019 g/ml	d < 1.019 g/ml	70–200 ml	210–400 ml
(a) Fat	2.0 ± 0.2^{b}	1.4	1.0	1.8
(b) 2% Chol	$1.2 \pm 0.2^{a,c}$	1.2	1.2	1.1
(c) 2% Chol + PTU	2.3 ± 0.5^{b}	3.0	1.4	5.4

Results are means ± 1 SD of dye uptake ratios for the indicated apoproteins separated by gradient SDS gel electrophoresis (3.5–20%). For the d < 1.019 g/ml column on the left, three to six plasma pools were examined; for the three columns on the right, single pools of ten rats each were analyzed (see Fig. 6). Letter superscripts indicate significant ($P \leq 0.05$) differences between dietary subgroups (see Table 1). Zonal effluent volumes (Fig. 3).

rather than triglycerides. Despite their similarities, the d < 1.019 g/ml lipoproteins and the zonal subfractions isolated from cholesterol + PTU-treated rats were much more readily taken up by cultured rat macrophages and fibroblasts than the analogous lipoproteins isolated from cholesterol-fed animals. In an attempt to account for these differences, the apoprotein compositions of the fractions were assessed by SDS-polyacrylamide gel electrophoresis (**Fig. 6**) and isoelectric focusing (not shown). ApoB, apoC, and apoE were the major apoproteins in the d < 1.019 g/ml fractions. Based on dye uptake ratios, $apoB_L$ was relatively more abundant than $apoB_S$ in the d < 1.019 g/ml fractions of all groups, but $apoB_L/apoB_S$ ratios of the Chol-fed groups were low compared with the fat-fed group (**Table 4** and **Table 5**). The higher $apoB_L/apoB_S$ ratios were found in Chol + PTU-fed rats, and the highest ratio was present in the 210–400 ml zonal effluent fractions of the Chol + PTU-treated rats. ApoE decreased relative to apoB and increased relative to apoC in Chol + PTU groups. ApoC/apoE ratios were particularly low in the 210–400 ml fractions (Table 5). These results are compatible with the diet- and PTU-induced redistributions of apoE into the VLDL fraction detected by gel permeation chromatography, and the low apoC/apoE ratios explain some of the slowed electrophoretic mobilities of the various d < 1.019 fractions.

DISCUSSION

Several complementary techniques were used to describe the lipoproteins of these animals, including fixed-angle and zonal ultracentrifugation, agarose electrophoresis, and gel permeation chromatography. In addition, the lipid and apoprotein compositions of selected lipoprotein fractions were described, using radioimmunoassay, SDS gel electrophoresis, and isoelectric focusing for quantifying the apoproteins.

Addition of 2% cholesterol to the 5% fat diet produced significant increases in plasma cholesterol and in d < 1.019 and d < 1.070 g/ml lipoprotein levels. ApoB levels rose while apoE and apoA-I fell. On zonal ultracentrifugation a slower floating VLDL species appeared and LDL was not present in its usual flotation position. LDL of rabbits also disappear from their usual flotation positions on high cholesterol diets (19). Others have reported increases in LDL under similar conditions (20, 21, 37). The latter workers noted LDL probably because

TABLE 5. Relative proportions of apoproteins in d < 1.019 g/ml lipoproteins

Diet	Lipoprotein	ApoC	ApoE	$ApoB_L$	$ApoB_S$	ApoC/ApoE	ApoE/ApoB
		% dye uptake				*dye uptake ratio*	
Fat	d < 1.019 g/ml	39	34	16	11	1.1 (1.0)	1.3
	70–200 ml	59	23	10	10	2.5	1.1
	210–400 ml	28	28	28	16	1.0	0.6
2% Chol	d < 1.019 g/ml	36	35	16	14	1.0 (0.9)	1.2
	70–200 ml	55	25	11	9	2.2	1.3
	210–400 ml	33	41	14	11	0.8	1.6
2% Chol + PTU	d < 1.019 g/ml	12	41	36	12	0.3 (0.2)	0.9
	70–200 ml	22	53	15	11	0.4	2.0
	210–400 ml	8	44	42	8	0.2	0.9

Results are dye uptakes on 3–20% SDS gradient gels. Data are expressed either as % of total dye uptake or as dye uptake ratios (Fig. 6). Results in parentheses are analogous results of scan of isoelectric focusing gels of a different group of animals. For the latter groups, coefficients of variation of apoC/apoE ratios ranged 11–21% for six plasma pools per diet group.

they centrifuged at fixed densities, which may have resulted in the flotation of IDL with the putative LDL. Cholesterol-induced VLDL was grossly enriched in cholesteryl esters at the expense of TG.

In contrast with the rabbit, where HDL virtually disappears on high cholesterol diets (19), in rats, HDL (d 1.070–1.21 g/ml) levels did not change and the zonal flotation rate of HDL or the column elution profiles of apoA-I also were not changed. However, on column chromatography, the apoE elution profile was altered from a peak elution between apoB and apoA-I to a profile where virtually all of apoE eluted in the VLDL range, suggesting that these diets by themselves did not increase HDL_1 or HDL_C levels of the usual size. It is possible that apoE-HDL_C (containing apoE but no apoA-I) of VLDL-IDL size did increase without our being able to detect it with the methods used.

The cholesterol plus PTU combination treatment produced greater increases of plasma cholesterol, apoB, apoE, and of d < 1.019 and d < 1.070 g/ml fractions. VLDL floated even more slowly and an IDL particle also was found, while LDL with usual flotation properties was absent. HDL levels and zonal effluent profiles were unchanged and on column chromatography apoE was distributed between the VLDL and HDL_C, indicating that PTU was necessary for the persistence of HDL_C of the usual size on high cholesterol diets.

The work of Dolphin (39) and Swift et al. (40) (who isolated nascent particles from the hepatic Golgi apparati of Chol + PTU-treated rats, which in their lipid compositions and electrophoretic mobilities resembled the d < 1.019 g/ml particles described above) suggests that the liver is a major source of these cholesterol-induced lipoproteins. Swift et al. (64) also have shown cholesterol enrichment of nascent VLDL isolated from enterocytes of similarly treated rats. Thus, some of the changes noted on Diet(c) can clearly be related to altered VLDL production by liver and intestine. Delayed catabolism may also play a role. Thyroid hormone lack delays clearance of chylomicron and VLDL remnants not by altering hepatic uptake capacity (65) but by "saturating" the "clearance" system, perhaps by reducing the activity of hepatic triglyceride lipase (66).

In the rat, as in other species (31), feeding of cholesterol produced d < 1.019 g/ml lipoproteins which stimulated [^{3}H]oleate esterification in cells to a much greater extent than the d < 1.019 g/ml lipoproteins isolated from fat-fed animals. In fact, it was difficult to demonstrate esterification above basal values, or lipoprotein dose-dependency of esterification with lipoproteins of fat-fed animals, while both effects were obvious with lipoproteins of Chol-fed rats. What was surprising to us was that addition of PTU to the cholesterol diet resulted in the production of lipoproteins that were even more stimulatory of [^{3}H]oleate esterification both in macrophages and fibroblasts. This was true whether whole d < 1.019 g/ml fractions or zonal subfractions 210–400 ml were compared. Since only normal cells were used in these studies and methods of lipoprotein isolations were identical, the differences in cell reactivity were due to the experimentally produced lipoproteins themselves. Several compositional and physical differences between cholesterol- and Chol + PTU-induced lipoproteins exist which may explain the differences in their cell reactivities. These include differences in size and net change, and altered $apoB_L$/$apoB_S$, apoC/apoE ratios. Sparks et al. (67, 68) have shown that $apoB_L$ and $apoB_S$-containing lipoproteins are removed from circulation at different rates, implying differential recognition by receptors. C-apoproteins, in particular apoC-III, modify recognition of apoE-containing lipoproteins by cells (69, 70). Thus, the changes in apoprotein contents of VLDL could account for some of the alterations of VLDL-cell interactions. Finally, changes in VLDL size, independently of or in combination with the changes of apoproteins, could affect "exposure" of cell recognition sites on lipoproteins (71) and thereby alter cell uptake. Since lipoproteins of similar flotation properties and lipid compositions were being compared, it is unlikely that different lipoprotein population mixtures present within the d < 1.019 g/ml fractions affected cell reactivity, nor did the differences in cholesterol contents of the lipoproteins explain the observations. Whatever the reasons for the difference, the in vitro lipoprotein-cell interaction studies are compatible with the well-known increased atherosclerosis-producing potential of the Chol + PTU diet regimens compared with Chol diets in vivo in this species (31). Thus, analysis of lipoprotein-cell interactions in vitro may help to explain the findings in vivo and the euthyroid state seems to provide important protection against atherogenic hyperlipidemia in the rat.

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