Apolipoprotein changes associated with the plasma lipid-regulating activity of gemfibrozil in cholesterol-fed rats

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Abstract Gemfibrozil (Lopid®) is a new plasma lipid-regulating drug that decreases very low and low density lipoprotein (VLDL/LDL) and increases high density lipoprotein (HDL) concentrations in man. The present experiments tested the effects of gemfibrozil on plasma lipoproteins and apolipoproteins in rats fed high fat/high cholesterol diets. Compared to chow-fed rats, cholesterol feeding for 2 weeks (20% olive oil/2% cholesterol) produced the expected increases in VLDL and intermediate density lipoprotein (IDL) while lowering plasma HDL. This was documented by using three methods of lipoprotein isolation: sequential ultracentrifugation, density gradient ultracentrifugation, and agarose gel filtration. Gemfibrozil gavaged at 50 mg/kg per day for 2 weeks during cholesterol feeding prevented these changes such that lipoprotein patterns were similar to those in chow-fed animals. Whole plasma apoE and apoA-I concentrations were decreased and apoB increased due to cholesterol feeding as determined by electroimmunoassay, but again gemfibrozil treatment prevented these diet-induced alterations. Gradient polyacrylamide gel electrophoresis patterns of the total d < 1.21 g/ml lipoprotein fractions reflected the changes in apolipoprotein concentrations and further demonstrated a greater increase of $apoB_l$ compared to $apoB_h$ in cholesterol-fed rats. Gemfibrozil lowered the concentration of both apoB variants and prevented the shift of apoE from HDL to lower density lipoproteins. Changes in the distribution of apoE were confirmed using agarose gel column chromatography followed by electroimmunoassay. These methods also revealed a shift of apoA-IV from HDL to the d > 1.21 g/ml, lipoprotein-free fraction with gemfibrozil treatment when blood was taken from fasted or postabsorptive animals. Since it was also noted that in chow-fed rats more apoA-IV was present in the d > 1.21 g/ml fraction in the postabsorptive or fed state compared to fasted animals, it could be postulated that the shift of apoA-IV into this fraction in gemfibrozil-treated rats is related to an accelerated clearance of chylomicrons. It is concluded that gemfibrozil largely prevents the accumulation of abnormal lipoproteins in this model of dyslipoproteinemia, and that apoE may play a critical role in this normalization process. —**Krause, B. R., and R. S. Newton.** Apolipoprotein changes associated with the plasma lipid-regulating activity of gemfibrozil in cholesterol-fed rats. *J. Lipid Res.* 1985. **26:** 940–949.

Supplementary key words lipoproteins • HDL • electrophoresis • apoB • apoE • apoA-I • apoA-IV

Gemfibrozil (Lopid®, **Fig. 1**) is a new agent for the management of dyslipoproteinemia. Extensive clinical investigations over the last 12 years, documented in several published symposia (1–3), indicate that gemfibrozil effectively lowers plasma triglyceride and increases HDL-cholesterol in both Type II and Type IV patients. The latter is especially important in view of the epidemiological evidence implicating HDL as being protective against coronary heart disease (CHD) (4, 5) and the recent data from primary (6, 7) and secondary trials (8, 9) indicating that increases in plasma HDL are associated with reduction of CHD risk. The Helsinki Heart Study is presently being conducted to test the hypothesis that increasing HDL-cholesterol by gemfibrozil treatment can decrease the incidence of CHD in a high-risk population (10). Since gemfibrozil generally decreases VLDL- and LDL-cholesterol while increasing HDL-cholesterol, decrements in plasma total cholesterol are usually less than 20% (11–13). Due to these changes in the distribution of cholesterol among lipoproteins, gemfibrozil can be considered a "lipid-regulating" in addition to a "hypolipidemic" agent. The purpose of the present study was to assess the effects of gemfibrozil treatment in rats fed high-cholesterol/high-fat diets. The results show that gemfibrozil can prevent the accumulation of abnormal lipoproteins in this model of dyslipoproteinemia, and further suggest that specific alterations in apolipoprotein metabolism may be involved in this lipid-regulating effect.

METHODS AND MATERIALS

Animals

Sprague-Dawley male rats (300–400 g) were fed a pel-

Abbreviations: VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LCAT, lecithin:cholesterol acyltransferase.

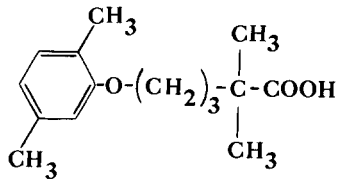

Fig. 1. Structure of gemfibrozil (Lopid®).

leted open-formula, nonpurified diet[1] (Purina Lab Chow No. 5001) with (cholesterol-fed) or without (chow-fed) supplementation with 20% olive oil and 2% cholesterol. The duration of all experiments was 2 weeks, and several experiments (five to seven rats/group) were conducted under identical conditions in order to study lipoproteins and apolipoproteins using the methods described in detail below. Gemfibrozil-treated rats received the olive oil/cholesterol diet and were dosed orally with gemfibrozil at 50 mg/kg per day. Control and cholesterol-fed rats were gavaged daily with drug vehicle alone (acacia). Animals were fasted 14 hr (midnight to 2 PM) and blood was obtained by heart puncture under ether anesthesia. Serum was separated at 12°C and subsequently preserved with 0.01% EDTA/sodium azide.

Lipoprotein isolation

Lipoproteins were isolated from serum either by sequential ultracentrifugation (14) or by density gradient ultracentrifugation (15) as indicated. For sequential isolation, very low density lipoproteins (VLDL) of d < 1.006 g/ml, intermediate density lipoproteins (IDL) of d 1.006–1.030 g/ml, and low density lipoproteins (LDL) of d 1.030–1.063 g/ml were separated at 39,000 rpm for 24 hr (10°C) in a Beckman Type 50 rotor with overlayering (1.3×10^8 *g*-min). High density lipoproteins (HDL) of d 1.063–1.21 g/ml were isolated under identical conditions for an additional 24 hr. For density gradient separation, 1.0 ml of serum was adjusted to 1.30 g/ml, layered with normal saline (d 1.006 g/ml), and centrifuged at 80,000 rpm for 50 min in a Beckman VTi80 rotor at 10°C. Lipoproteins were visualized with Sudan Black B and densities were checked directly on all lipoprotein fractions using a density meter (DMA-35, Mettler/Paar, Graz, Austria). All lipoprotein fractions were dialyzed exhaustively against 0.9% NaCl containing 0.01% EDTA-sodium azide prior to electrophoresis.

In some experiments a total lipoprotein fraction (d < 1.21 g/ml) was obtained by adjusting plasma to d 1.24 g/ml, layering with KBr solution of d 1.21 g/ml (at least 50% of total tube volume), and centrifuging 48 hr in a Beckman Type 50 rotor at 39,000 rpm (2.65×10^8 *g*-min). This total lipoprotein fraction was dialyzed as above and then subfractioned by gel filtration chromatography (Bio-Gel, A-5m, Bio-Rad Labs, Richmond, CA) as previously described (16) using a 2.6 × 100 cm column (A-5m) equilibrated and eluted with 0.15 M NaCl, pH 7.4. Fractions of 5 ml were collected (Gilson FC-100 Fractionator, Gilson Electronics, Middleton, WI). Lipoproteins were identified by comparison to the elution of human LDL, albumin, and ultracentrifugally isolated rat lipoproteins as indicated in the legends. Void volume was determined using Blue Dextran 2000.

Electrophoresis

The apolipoprotein composition of lipoproteins was determined by polyacrylamide gel electrophoresis in the presence of SDS. Ultrafiltration membrane cones (Centriflo CF-25, Amicon Corp., Lexington, MA) were used to concentrate IDL prior to electrophoresis. Lipoproteins were partially delipidated by vortexing twice with cold ether (20 volumes) and then solubilized in 0.0625 M Tris-HCl (pH 6.8) containing 5% SDS, 30% glycerol, and 0.03% bromophenol blue. Samples were then reduced by heating at 100°C for 2 min with 1/10 volume of 400 mM dithiothreitol. Electrophoresis was performed at room temperature using 3–20% gradients in a vertical slab unit (Hoeffer Instruments, San Francisco, CA) at a constant current of 10 mA/slab. The buffers and SDS concentrations were exactly as described by Laemmli (17). Gels were stained with 0.1% Coomassie Brilliant Blue G-250 in 50% methanol–10% glacial acetic acid (6 hr at 23°C) and destained in 10% acetic acid overnight. Percentage composition of lipoproteins was determined by linear densitometric scanning (Helena Laboratories, Beaumont, TX). Molecular weight standards were from Bio-Rad Laboratories (Richmond, CA).

Apolipoprotein concentrations in whole serum were determined by electroimmunoassay (18). In order to increase the exposure of antigenic determinants, serum was diluted in 0.1 M Tris-HCl, pH 8.0, containing 1% deoxycholate, 0.15 M NaCl, 1% Triton X-100, and 0.1% SDS (R. A. Davis, unpublished observations). When apolipoprotein concentrations were determined in fractions from the gel filtration column, NP-40 (0.05%) was added to the fractions prior to electroimmunoassay. Samples were applied to antibody-containing plates[2] of 2% (apoA-I,

[1] The designation "nonpurified" is used to refer to diets composed predominantly of unrefined plant and animal materials. "Open formula" indicates that the precise percentage composition of each ingredient is available and that no changes in the type or amount of ingredients are permitted. The use of these terms has been recommended by the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies (1977. *J. Nutr.* **107:** 1340–1348).

[2] Monospecific, polyclonal antibodies raised in goats against rat apoE, A-I, A-IV, and B were prepared in New Orleans and kindly supplied by Dr. Paul Roheim and colleagues, Louisiana State University Medical Center. Antisera were shipped and stored at 4°C with 0.1% EDTA-sodium azide, 0.01% merthiolate, and 0.05% phenol. The antisera for apoB reacted equally against rat $apoB_h$ and $apoB_l$ as determined by immunoblotting onto cellulose nitrate sheets (L. Wong, unpublished data).

apoE), 1.2% (apoA-IV), or 1% (apoB) agarose (SeaKem ME, FMC Corp., Rockland, ME) in 0.025 M Tris-tricine buffer, pH 8.6, containing 34 mM calcium lactate and 0.1% sodium azide. Dextran (average mol wt 63,200) was also added to the agarose at 3% (apoA-I, apoE) or 2% (apoA-IV) to prevent migration and "blurring" of the precipitates. Polyethylene glycol (3%) was used for the apoB plates. Electrophoresis was performed in a horizontal apparatus (Model 1405, Bio-Rad Labs, Richmond, CA) at 16°C at a field strength of 1.7–2.0 volts/cm for 16–18 hr. The gels containing the immunoprecipitates were washed in saline followed by water, and then dried on top of a sheet of clear graphic arts film (Cronar C-42, DuPont, Wilmington, DE). The film containing the transferred immunoprecipitates was stained with Coomassie Brilliant Blue R-250 (0.05% in methanol–water–acetic acid 75:75:15, v/v/v) and destained in the same solution without dye. A reference serum was used in multiple dilutions as a secondary standard (19). This serum was assayed against purified apolipoproteins (primary standards)[3] and stored at −70°C.

Lipid determinations

Total cholesterol and triglyceride concentrations in serum were determined by standard AutoAnalyzer II methodology (20). In some experiments, aliquots of serum were precipitated with $MnCl_2$ and heparin to estimate HDL-cholesterol (21). Total protein in lipoprotein fractions was measured by the method of Lowry et al. (22). Statistical differences between mean values (Tables 1 and 2) were determined by Student's *t*-test.

RESULTS

Serum lipids and lipoproteins

Although the high-fat/high-cholesterol diet resulted in only a mild hyperlipidemia (increased triglycerides and cholesterol by 58% and 29%, respectively), it dramatically altered the distribution of both cholesterol and protein among the serum lipoproteins (**Table 1**). Specifically, cholesterol feeding decreased HDL-cholesterol (58%) and protein (40%) while increasing the concentrations of these constituents in lower density lipoproteins. In chow-fed rats most cholesterol (61%) and protein (80%) was in the HDL fraction, but with cholesterol feeding these figures were reduced to 19% and 50%, respectively, and IDL-protein increased to represent 32% of total lipoprotein-protein. Gemfibrozil dosed orally during cholesterol feeding prevented these changes such that HDL-cholesterol and protein concentrations were higher and VLDL/IDL-protein and serum triglyceride concentrations were lower compared to cholesterol-fed animals not receiving gemfibrozil. In fact, all of the parameters presented in Table 1 for cholesterol-fed rats dosed with gemfibrozil are similar to those in chow-fed rats. These results could not be explained on the basis of decreased food intake since body weight gain was not significantly different among the three treatment groups (mean body weight gain was 15.1% in repeated experiments).

The changes in lipoprotein cholesterol were easily illustrated by density gradient ultracentrifugation. This rapid, single-spin technique, which allows visualization of lipid-stained lipoproteins, also demonstrated a decrease in the concentration of HDL (band B) and accumulation of lower density lipoproteins (floating material, band A) in cholesterol-fed rats (**Fig. 2**). These density gradient profiles for both normal and cholesterol-fed rats are similar to those reported by Terpstra, Van Tintelen, and West (23). In some chow-fed rats, two HDL bands were apparent (not shown), presumably corresponding to HDL_1 and HDL_2 (24, 25). When present, the denser of the two HDL bands always predominated in both chow-fed and in cholesterol-fed rats receiving gemfibrozil. It was also apparent by using this technique (Fig. 2) that gemfibrozil prevented the loss of HDL and the accumulation of large, lipid-rich particles during cholesterol feeding.

Finally, a total lipoprotein fraction ($d < 1.21$ g/ml) was isolated by ultracentrifugation and subjected to gel filtration chromatography to separate lipoproteins. Although overestimated due to light scattering, large particles in the void volume, corresponding in size to VLDL and IDL, were increased in concentration due to cholesterol feeding and there was a decrease in the appearance of smaller lipoproteins which co-eluted with ultracentrifugally isolated HDL (**Fig. 3**). Both of these alterations were prevented by gemfibrozil treatment. Lipoproteins corresponding in size to LDL (fractions 46–50) were not detected under these conditions and often do not appear as a distinct peak (26). Thus, sequential ultracentrifugation (Table 1), density gradient ultracentrifugation (Fig. 1), and agarose gel chromatography (Fig. 2) all demonstrate essentially the same results regarding the effects of cholesterol feeding and gemfibrozil on the distribution of lipid and protein among the serum lipoproteins.

Apolipoprotein concentration

The changes due to cholesterol feeding in the whole serum concentrations of apolipoproteins E, A-I, and B were similar to those reported previously for longer-term experiments (27, 28). ApoE and apoA-I concentrations decreased by approximately 60% and 15%, respectively, while apoB concentrations increased twofold (**Table 2**). Gemfibrozil treatment prevented the decreases in apoE

[3] We are indebted to Christine Castle, LSU Medical Center, for these determinations. The concentrations in our reference serum were 6.8, 16.2, 35.0, and 34.5 mg/dl for apoA-IV, B, E, and A-I, respectively.

TABLE 1. Effect of gemfibrozil on serum lipids and lipoproteins

	Chow-Fed	Cholesterol-Fed	Cholesterol-Fed + Gemfibrozil[a]
Lipids (mg/dl)			
Triglyceride	35.3 ± 1.5 (6)	55.8 ± 4.5[b] (6)	32.8 ± 2.2 (6)
Cholesterol	64.3 ± 2.9 (6)	82.7 ± 3.3[b] (6)	71.2 ± 7.3 (5)
HDL-cholesterol[c]	39.0 ± 2.0 (6)	16.2 ± 0.9[b] (6)	33.8 ± 3.2 (5)
Lipoproteins (mg protein/dl)[d]			
VLDL (d 1.006 g/ml)	3.7 [6.7][e]	6.7[b] [12.2]	2.8 [5.5]
IDL (d 1.006-1.030 g/ml)	2.4 [4.3]	17.6[b] [32.0]	3.2 [6.3]
LDL (d 1.030-1.063 g/ml)	5.0 [9.1]	4.5 [8.2]	3.5 [6.9]
HDL (d 1.063-1.21 g/ml)	43.7 [79.7]	26.2[b] [47.6]	41.5 [81.4]

Values are the mean ± SEM. Numbers in parentheses are the number of rats per group.
[a]Gemfibrozil was dosed orally at 50 mg/kg per day for 2 weeks.
[b]Significantly different from chow-fed value, $P < 0.05$.
[c]Measured by heparin-Mn^{2+} precipitation (21).
[d]Isolated by sequential ultracentrifugation (14). Values are the means of two or three serum pools (10 rats per pool).
[e]Numbers in brackets refer to precent of total lipoprotein-protein.

and apoA-I and the increase in apoB concentrations during cholesterol feeding. Although numerically greater in cholesterol-fed rats, whole serum apoA-IV concentrations were always the most variable and were not significantly altered by drug or diet after the 2-week period.

Apolipoprotein composition

The apolipoprotein composition of centrifugally isolated lipoproteins was examined using SDS-polyacrylamide gel electrophoresis. Volumes were kept constant between groups throughout the centrifugation and electrophoresis procedures. Therefore, the staining intensity of the bands qualitatively reflects differences in apolipoprotein concentration in all of the gels presented below. Initially, an experiment was conducted to determine the apolipoprotein composition of the entire d < 1.21 g/ml fraction (**Fig. 4**). In chow-fed rats, apoE and apoA-I represented 37% and 45% of the total d < 1.21 apolipoproteins, respectively. The remainder included primarily $apoB_l$, $apoB_h$, and apoC. With cholesterol feeding, apoE represented only 14% and apoA-I more than 54% of the total protein. The percentage of apoB increased from 5% to 20% with cholesterol feeding, with most of the increase due to $apoB_l$. When cholesterol-fed rats were dosed with gemfibrozil, the percentage compositions resembled those in chow-fed rats (36% for apoE, 40% for apoA-I, and 12% for apoB). The relative staining intensities as visualized in Fig. 4 are consistent with the electroimmunoassay data in that the lowering of both apoE and the increase in apoB concentrations, especially $apoB_l$, are prevented by gemfibrozil in cholesterol-fed rats. ApoC is not well resolved in this system and was not evaluated in this study.

A comparison of the VLDL, IDL, and LDL gel patterns from 3–20% gradients are shown in **Fig. 5**. In VLDL from chow-fed animals, $apoB_h$, $apoB_l$, and apoE represented 15%, 30%, and 60% of total protein, respectively. Cholesterol feeding increased the percentage of $apoB_l$ and $apoB_h$ to 34% and 20%, respectively, and decreased the percentage of apoE to 46%. In gemfibrozil-treated rats, the percentage compositions were the same as in chow-fed animals. Despite the relatively minor changes in the percentage compositions of VLDL, visual inspection of staining intensities are consistent with the data presented above (Table 1 and Figs. 2 and 3) in that there is clearly more protein in the VLDL from cholesterol-fed rats and approximately equal amounts of apoB and apoE in chow-fed and gemfibrozil-treated rats. The relative concentrations of both apoB subspecies in VLDL were increased with cholesterol feeding, as occurs after a single

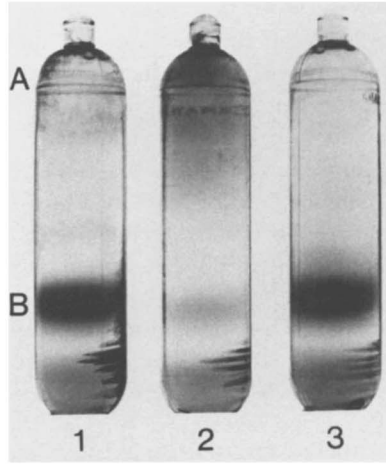

Fig. 2. Separation of lipoproteins by density gradient ultracentrifugation. Whole plasma (1.0 ml) was adjusted to d 1.030 g/ml, placed into polyallomer centrifuge tubes (13 × 51 mm), and centrifuged for 50 min in a VTi80 rotor. Tubes 1, 2, and 3 are from chow-fed, cholesterol-fed, and cholesterol-fed plus gemfibrozil groups. Band A, VLDL + IDL and band B, HDL, as determined by direct density measurements (A, 1.030 g/ml and B, 1.073–1.100 g/ml).

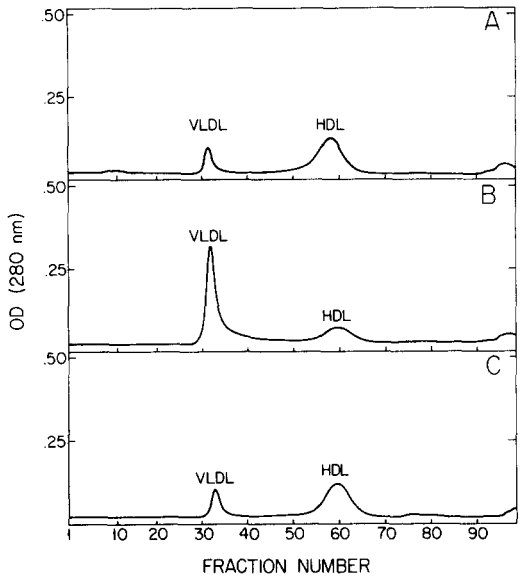

Fig. 3. Separation of the total d < 1.21 g/ml fraction by gel filtration chromatography (Bio-Gel A-5m) from chow-fed (A), cholesterol-fed (B), and cholesterol-fed plus gemfibrozil (C) rats. Lipoproteins were obtained from 5 ml of whole plasma. Running conditions are described in the text. "VLDL" and "HDL" were identified by comparison to the elution of ultracentrifugally isolated lipoproteins, human LDL (fractions 46–50), and albumin (fractions 73, 74).

meal of the same diet (29), and both variants were lowered by gemfibrozil treatment. Cholesterol feeding increased the concentration of IDL-protein by approximately 10-fold (Table 1), and this was reflected in the qualitative electrophoretic separation of IDL apolipoproteins (lanes 5–7). The resultant composition of IDL after gemfibrozil treatment resembled that of normal IDL. The amounts of LDL protein were not significantly different among the groups (Table 2), but in chow-fed and gemfibrozil-treated rats $apoB_h$ was the major protein, while in LDL from cholesterol-fed rats approximately equal amounts of the two apoB variants were present (i.e., relative enrichment of LDL with $apoB_l$ in cholesterol-fed rats).[4]

The HDL composition is shown in **Fig. 6**. HDL was isolated for electrophoresis by a single density gradient spin rather than by sequential ultracentrifugation in order to avoid losses of apolipoprotein. Therefore, considerable amounts of albumin are present. Three representative rats per group are illustrated. The percentages of apoA-I (50–60%) and apoE (20–25%) were very similar in chow-fed and gemfibrozil-treated groups but, in cholesterol-fed rats not receiving gemfibrozil, apoA-I became the major apolipoprotein in HDL (74%) since very little apoE was

[4] The band in LDL and in Fig. 4 between $apoB_h$ and $apoB_l$ was not further characterized and could be a proteolytic product of $apoB_h$ as described by Cardin et al. (1984. *Federation Proc.* **43**: 1532, abstract).

TABLE 2. Effect of gemfibrozil on whole serum apolipoprotein concentrations

	Chow-Fed	Cholesterol-Fed	Cholesterol-Fed + Gemfibrozil
		mg/dl	
ApoE	36.4 ± 2.6	13.4 ± 1.0[a]	38.6 ± 2.0
ApoA-I	39.5 ± 0.5	33.5 ± 1.3[b]	42.5 ± 2.4
ApoB	25.9 ± 0.6	63.9 ± 2.0[a]	22.1 ± 1.1
ApoA-IV	6.8 ± 0.2	9.2 ± 1.7	8.7 ± 0.3

Values are the mean ± SEM of five to seven rats/group. Gemfibrozil was dosed orally at 50 mg/kg per day for 2 weeks.
[a] Significantly different from chow-fed group, $P < 0.001$.
[b] $P < 0.05$.

present. Thus, these gels illustrate that the decrease in whole plasma apoE (Table 2) is perfectly reflected in the HDL fraction and prevented by treating cholesterol-fed rats with gemfibrozil. The changes in apoA-I are qualitatively similar to and consistent with those for whole plasma using electroimmunoassay (Table 2) in that there were slight decreases of apoA-I in cholesterol-fed animals not receiving gemfibrozil.

Apolipoprotein E and A-IV distribution

The apolipoprotein that was altered to the greatest extent in terms of both concentration (Table 2) and lipoprotein composition (Figs. 4 and 6) was apoE. Therefore, the distribution of apoE was further examined without ultracentrifugation using a combination of electroimmunoassay and gel filtration. Using these techniques, a dramatic decrease of apoE in HDL-sized particles was evident due to cholesterol feeding, whereas with gemfibrozil treatment the content of apoE was actually greater in these lipoproteins compared to chow-fed animals (**Fig. 7**, panel B). Therefore, the percentage of total apoE in lipoproteins eluting in the void volume increased dramatically in cholesterol-fed animals (panel A), and the percentages of apoE in the two regions corresponding in size to VLDL and HDL for cholesterol-fed rats treated with gemfibrozil were very similar to those in chow-fed rats. These techniques confirm the results from SDS-PAGE indicating an apparent shift in apoE distribution in cholesterol-fed rats not receiving gemfibrozil.

Whole plasma apoA-IV concentrations were slightly higher but variable in cholesterol-fed rats compared to chow-fed animals, yet the apolipoprotein composition of HDL, the major apoA-IV-containing lipoprotein, suggested a slight decrease in apoA-IV (Fig. 6). To investigate this further, apoA-IV was determined in fractions corresponding to HDL or to the d > 1.21 g/ml lipoprotein-free fraction after gel filtration of whole serum. Serum was placed on the column immediately after the animals were killed, since preliminary data indicated that sample storage at 4°C altered the distribution of apoA-IV. In

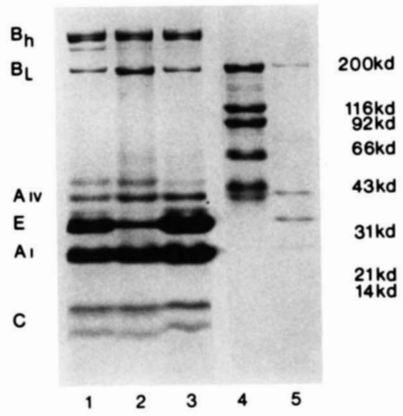

Fig. 4. Electrophoretic separation of a total d < 1.21 g/ml lipoprotein fraction from chow-fed (lane 1), cholesterol-fed (lane 2), and cholesterol-fed plus gemfibrozil (lane 3) rats. Comparison is made to molecular weight standards (lane 4) and to mesenteric lymph chylomicrons (lane 5), the latter containing only the low molecular weight variant of apoB (50).

addition, the experiments reported herein using a relatively short fast (i.e., postabsorptive) were compared to 24-hr fasted rats and to fed animals inasmuch as the distribution of apoA-IV between HDL and the lipoprotein-free fraction is related to chylomicron metabolism (30). In all groups, the distribution of apoA-IV was dependent upon the nutritional state. Most apoA-IV in fasted, chow-fed rats was associated with HDL (79%), while in post-absorptive and fed rats apoA-IV was distributed equally (**Table 3**). When blood was taken from fasted, cholesterol-fed rats, a shift of apoA-IV to the lipoprotein-free fraction was noted, but this change was much less pronounced in postabsorptive or fed rats. Gemfibrozil resulted in the appearance of more apoA-IV in the lipoprotein-free fraction in fasted and post-absorptive animals but not in the fed state.

DISCUSSION

The abnormal lipoproteins produced by cholesterol feeding in rats have been well characterized. High-fat/cholesterol diets in particular cause apparent shifts in the lipid and protein constituents from high to lower density lipoproteins as evidenced by increases in VLDL/IDL and decreases in HDL (27, 28, 30–35). Cultured hepatocytes from such animals accumulate and secrete large amounts of VLDL lipids (36), and cholesterol-rich IDL are secreted into the intestinal lymph at increased rates (37), suggesting that both the liver and intestine may contribute to the observed alterations in plasma lipoproteins. Apolipoprotein changes in the present study which are consistent with previous reports using this dyslipoproteinemic model (27, 28) include *1*) decreases in whole plasma apoA-I concentrations; *2*) increases in apoB concentrations, especially apoB$_l$; *3*) essentially a loss of apoE from HDL; *4*) an enrichment of VLDL and IDL with apoE; and *5*) an apparent shift of apoA-IV to the lipoprotein-free fraction.

When cholesterol-fed rats were dosed daily with gemfibrozil, the resultant lipoprotein and apolipoprotein profiles resembled in many respects those of chow-fed rats; that is, gemfibrozil prevented the accumulation of abnormal lipoproteins. The observations concerning apoE are especially interesting in view of the documented role of this apolipoprotein in the hepatic recognition and uptake of circulating lipoproteins. Specifically, it has been suggested, from examining the lipoproteins from both the plasma (38) and peripheral lymph (i.e., interstitial fluid) (39, 40) of cholesterol-fed animals, that apoE may direct peripheral cell cholesterol to hepatic receptors for removal through the biliary system. In the cholesterol-fed rat it is possible that apoE concentrations in whole plasma, and especially in HDL, decrease due to a saturation of these removal mechanisms in the face of a high dietary fat/cholesterol load. The removal of chylomicron remnants (41) and IDL (42) is probably also mediated by apoE, thus possibly contributing to the decrease in plasma apoE pool size. We previously suggested that the loss of apoE in HDL could also reflect increased transfer of apoE from HDL to chylomicrons during the feeding of the high-fat/high-cholesterol diet, with subsequent removal of apoE during chylomicron uptake by the liver (43). Regardless of the mechanisms involved for the decrease in apoE concentrations, it is clear from the present study that gemfibrozil prevents this loss of apoE, and this apparent normalization of plasma apoE concentrations is associated with lipoprotein lipid patterns and apoE distribution which are similar to those in chow-fed animals. Such data would appear to give support for the critical role of apoE in normal lipoprotein metabolism (38, 42). Whether gemfibrozil treatment in man results in similar changes in

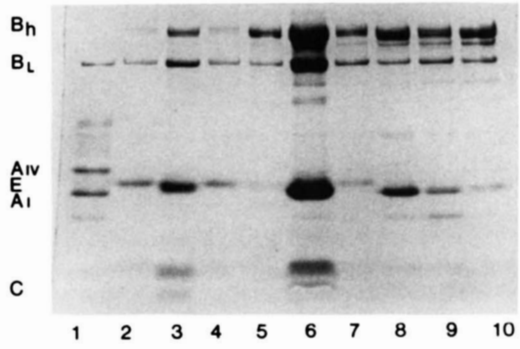

Fig. 5 Electrophoretic separation of VLDL (lanes 2–4), IDL (lanes 5–7), and LDL (lanes 8–10) from chow-fed (lanes 2, 5, 8), cholesterol-fed (lanes 3, 6, 9), and cholesterol-fed plus gemfibrozil (lanes 4, 7, 10) rats. Chylomicrons, which contain only apoB$_l$ (50), are again shown for comparison (lane 1).

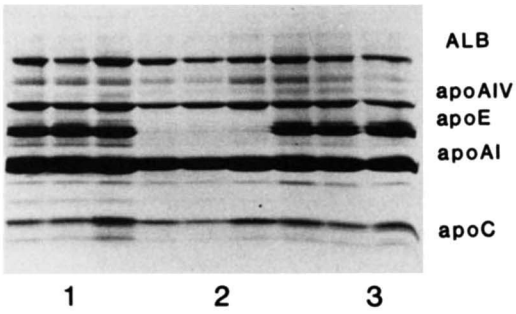

Fig. 6. Electrophoretic separation of HDL (d 1.063–1.21 g/ml, 3–20% polyacrylamide gradient) from chow-fed (1, lanes 1–3), cholesterol-fed (2, lanes 4–6), and cholesterol-fed plus gemfibrozil (3, lanes 7–9) rats. HDL was isolated by density gradient ultracentrifugation from three separate rats per group.

apoE has not been studied, but it should be mentioned that in normal man diets high in fat and cholesterol also shift apoE from high to lower density lipoproteins (44), and the clearance of triglyceride after heparin administration in hypertriglyceridemic patients apparently shifts apoE from VLDL back to HDL (45).

When rats are chronically fed powdered, purified diets containing cholesterol and cholic acid, the relative amount of $apoB_l$ increases in lipoproteins, even in HDL (46, 47). This also occurs in the IDL of rats after a single olive oil/cholesterol meal (29), and was apparent in the present experiments (Figs. 4 and 5) after chronic feeding of the same diet using density gradient SDS-PAGE, an electrophoretic system reported to give suitable separations of apoB variants (48). Since $apoB_l$ is produced by both the liver and intestine in the rat (49, 50), it does not serve as a marker for intestinal particles as it does in man (51, 52). Therefore, the origin of the "excess" $apoB_l$ in lipoproteins from cholesterol-fed rats and the reasons for the apparent prevention of changes in apoB heterogeneity due to gemfibrozil treatment cannot be ascribed to either the liver or intestine based solely on plasma data. The lack at the present time of immunological methods to separately quantitate these proteins in the rat is a major obstacle in this regard, and it should be emphasized that in the present experiments we only visually estimated $apoB_l$ and $apoB_h$ concentrations using SDS-PAGE. Although SDS-PAGE provides useful qualitative information on apoB subspecies concentrations (47), it assumes similar chromogenicities for the apoB variants and quantitative resolubilization of the variants after delipidation, both of which remain to be proven in future studies.

The hydrolysis of triglyceride-rich lipoproteins (chylomicrons and VLDL) by lipoprotein lipase (LPL) results in the transfer of surface components to HDL, thus contributing to the size of the HDL pool (53). It is feasible that the decrease of VLDL and increase of HDL found in the present experiments in cholesterol-fed rats treated with gemfibrozil is due in part to a transfer of VLDL surface remnants to the HDL density range. This would imply that under the present conditions lipoprotein lipase activity may also be elevated by gemfibrozil,[5] as reported for hypertriglyceridemic human subjects treated with gemfibrozil (54, 55). To what extent increases in HDL-cholesterol represent addition of VLDL surface material, peripheral cell cholesterol (reverse cholesterol transport), or cholesterol from other sources remains to be determined. Similarly, the extent to which changes in apoE distribution, as noted above, are causally related to the reciprocal relationship between VLDL and HDL is not known.

Among the plasma lipoproteins, apoA-IV in the rat is mainly found in the HDL fraction (56). With longer-term cholesterol feeding, (4 weeks), it has been reported that whole plasma apoA-IV concentrations increase by 40% (28). In the present experiments of only 2 weeks duration, apoA-IV increased nonsignificantly by 20% (Table 2). In chow-fed rats most apoA-IV was found in HDL when blood was obtained in the fasted state, but in postabsorptive or fed animals apoA-IV was equally distributed between HDL and the lipoprotein-free fraction. Cholesterol feeding altered the apoA-IV distribution to the greatest extent when blood was obtained from fasted rats, in which case more apoA-IV was found in the lipoprotein-free fraction. Gemfibrozil induced a further shift of apoA-IV from HDL to the lipoprotein-free fraction, but only when blood was taken from postabsorptive or fasted rats. It has been postulated that the transfer of apoA-IV to HDL is due to the activity of LCAT (57), and that this transfer is decreased by cholesterol feeding, resulting in more apoA-IV in the lipoprotein-free fraction (ref. 28 and Table 3). We have found the greatest percentages of apoA-IV in HDL in plasma from fasted, chow-fed rats and increased amounts of apoA-IV in the lipoprotein-free fraction with cholesterol feeding, consistent with increased LCAT (% of [^{14}C]cholesterol esterified/hr) with starvation and decreased LCAT with cholesterol feeding (58). In addition, storage of plasma results in a shift of apoA-IV to HDL, (B. R. Krause and R. S. Newton, unpublished data), possibly due to LCAT activity. Movement of apoA-IV in the opposite direction from HDL to the lipoprotein-free form may be related to the clearance of chylomicrons or to lipoprotein lipase activity (J. DeLamatre and P. S. Roheim, personal communication) since more apoA-IV in chow-fed rats is found in the lipoprotein-free fraction in postabsorptive or fed rats. Gemfibrozil further increased the amount of apoA-IV in the lipoprotein-free fraction, but only in postabsorptive or fasted animals. It can be specu-

[5]Preliminary evidence indicates that gemfibrozil increases LPL activity in rat adipose tissue and heart (A. D. Hartman and B. R. Krause, unpublished data).

lated that the accelerated clearance of chylomicrons by gemfibrozil after a meal (postabsorptive and fasted) results in the shift of apoA-IV to the lipoprotein-free fraction, but in fed animals not enough chylomicrons have been metabolized since most food is still in the stomach. The physiological significance of "free" apoA-IV is uncertain, but recently it has been suggested that it may participate in cholesterol removal from peripheral tissues (39). Further work is required, however, to establish the precise role of apoA-IV in this process and even more to determine the clinical significance of drug-related changes in apoA-IV distribution.

ApoA-I concentrations in whole plasma are lowered by cholesterol feeding, and this is due entirely to a decrease in HDL-apoA-I (28). The present experiments confirm this finding and demonstrate that these changes are prevented by gemfibrozil treatment. In vitro observations indicating the capacity of HDL-apoA-I to promote cholesterol efflux from cells (59, 60) and case-control studies in man indicating lower plasma apoA-I in patients with ischemic heart disease (61) both suggest that elevation of apoA-I is a potentially beneficial effect for a lipid-regulating drug.

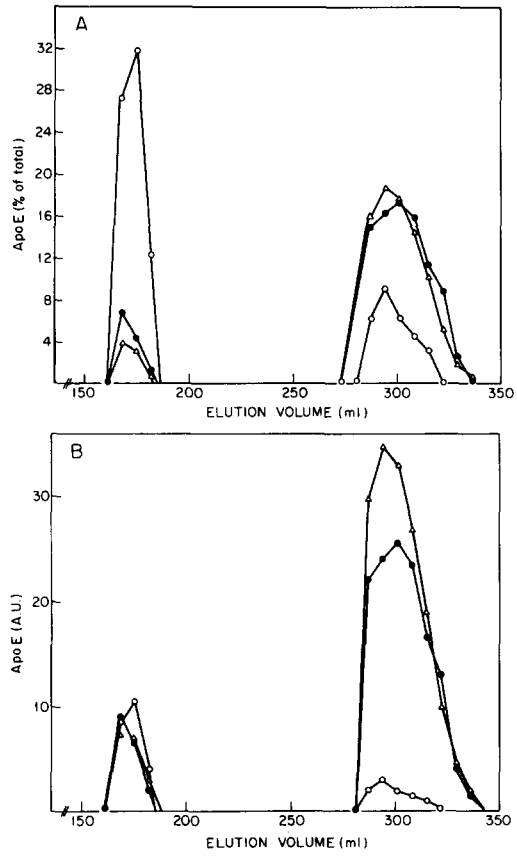

Fig. 7. Elution profile for apoE, expressed both as a percentage of the total (panel A) and the absolute amount/fraction (panel B) from chow-fed (●), cholesterol-fed (○), and cholesterol-fed plus gemfibrozil (△) rats. One ml of plasma from each of five rats/group was loaded (total 5 ml) and electroimmunoassay was performed directly on eluted fractions after addition of NP-40 (0.05%). Conditions for gel filtration were exactly as in Fig. 3.

TABLE 3. Effect of gemfibrozil on the percentage distribution of apoA-IV[a]

	Fasted	Postabsorptive	Fed
Chow-fed	79/21	52/48	54/46
Cholesterol-fed	50/50	46/54	46/54
Cholesterol-fed + gemfibrozil	33/66	31/69	68/32

One ml of serum from each of five animals was pooled (5 ml total) and fractionated on Bio-Gel A-5m immediately after the rats were killed. Electroimmunoassay was then performed directly on individual column fractions and rocket heights were measured in the "HDL" region (fractions 55–59) and in fractions eluting with or after albumin (lipoprotein-free region, fractions 73–84). Fasted rats were deprived of food for 24 hr. Postabsorptive animals had their food removed at midnight and were killed at 1 PM (see Methods and Materials). Fed animals were killed between 7 and 8 AM.

[a]Values are given as % in HDL/% in lipoprotein-free fraction.

It should be emphasized that other drugs resembling gemfibrozil in structure (phenoxy-alkanoic acids) do not possess similar activities in cholesterol-fed rats. For example, in our laboratory gemfibrozil but not bezafibrate prevents the decreases in plasma HDL and the accumulation of liver cholesterol in rats fed cholesterol/peanut oil diets (62). Gemfibrozil but not clofibrate is also active (i.e., increases HDL) in rats fed a cholesterol/peanut oil/cholic acid diet (63). In addition, gemfibrozil (and cholestyramine) but not bezafibrate or clofibrate cause an apparent stimulation of liver cholesterogenesis as measured in vivo using labeled octanoate (62). Thus, gemfibrozil (Lopid®) has effects distinctly different from either bezafibrate or clofibrate in rats, consistent with the differences between gemfibrozil and clofibrate observed in man (64).

In conclusion, gemfibrozil prevents the accumulation of abnormal lipoproteins in cholesterol-fed rats. In fact, the concentrations and apolipoprotein compositions of the plasma lipoproteins from cholesterol-fed rats dosed with gemfibrozil resemble those found in normal, chow-fed rats. The most obvious drug-related effect on apolipoproteins is the prevention of the loss of apoE in cholesterol-fed rats, which could be due to a decrease in apoE catabolism or an increase in apoE synthesis in the periphery or in the liver (65). The increase of apoB (B_l and B_h) by cholesterol feeding is also prevented by gemfibrozil treatment. Future experiments are designed to explore possible mechanisms responsible for the apparent changes in apolipoprotein metabolism induced by gemfibrozil.

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