

## Abnormalities in Very Low, Low, and High Density Lipoproteins in Hypertriglyceridemia

### Reversal toward Normal with Bezafibrate Treatment

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**A**bstract. The effects of triglyceridemia on plasma lipoproteins were investigated in 16 hypertriglyceridemic (HTG) subjects (222–2,500 mg/dl) before and after the initiation of bezafibrate therapy. Bezafibrate caused a mean reduction of 56% in plasma triglyceride and increased the levels of lipoprotein and hepatic triglyceride lipases by 260 and 213%, respectively. The natures of very low density lipoprotein (VLDL), isolated at plasma density and of low and high density lipoprotein (LDL and HDL), separated by zonal ultracentrifugation, were determined. HTG-LDL appears as multiple fractions whereas HTG-HDL is seen predominantly as HDL<sub>3</sub>.

HTG-VLDL is relatively poor in apoproteins and triglycerides but enriched in free and esterified cholesterol. HTG-LDL (main fraction) is depleted of free and esterified cholesterol but enriched in apoprotein and triglyceride. It is also denser and smaller than normal. HTG-HDL<sub>3</sub> is denser than *N*-HDL<sub>3</sub> and demonstrates compositional abnormalities similar to those of HTG-LDL. With the reduction of the VLDL mass, all abnormalities revert towards normal. This is accompanied by an increase in LDL-apoprotein B and cholesterol levels, which indicates an increased conversion of VLDL to LDL.

Significant correlations between plasma triglyceride and the degree of all abnormalities are shown. The data obtained during treatment corroborate these relationships. The observations support the concept that most abnormalities reflect the degree of triglyceridemia.

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We suggest that plasma core-lipid transfer protein(s) is an effector of the abnormal cholesteryl ester distribution. Its prolonged action on increasingly large and slowly metabolized VLDL populations would entail a correspondingly excessive transfer of cholesteryl ester to VLDL and of triglyceride to LDL and HDL. It is calculated that, in moderate HTG, LDL and HDL contain only 50% of the normal cholesterol load. It is suggested that cholesteryl ester redistribution in HTG might be important in regulating metabolic events.

### Introduction

Studies carried out during the last decade have demonstrated several metabolic pathways that affect all plasma lipoprotein classes. One such pathway is the activity of the lipoprotein lipase system, which results not only in triglyceride (TG)<sup>1</sup> hydrolysis but also in the formation of intermediate and low density lipoprotein (IDL and LDL) (1, 2). This same activity contributes lipids and proteins to the high density lipoprotein (HDL) (1–5) and may be essential for the formation in plasma of the lighter and larger subpopulation HDL<sub>2</sub> (6). Another pathway involves cholesterol esterification by lecithin/cholesterol acyltransferase (7) and the redistribution of cholesteryl esters (CEs) among the different lipoproteins, a reaction mediated by specific lipid transfer proteins (8–11). These proteins are also responsible for the redistribution of TG from TG-rich lipoproteins to LDL and HDL (12). Analysis of the potential effects of these and other metabolic events predicts that lipoprotein composition, structure, and metabolism should differ among subjects with various forms of dyslipoproteinemia, in particular hypertriglyceridemia (HTG). Indeed, in severe forms of HTG such as the

1. Abbreviations used in this paper: apo A-I and apo B, apoproteins A-I and B; C, cholesterol; CE, cholesterol ester; HTG, hypertriglyceridemia, hypertriglyceridemic; NTG, normotriglyceridemia, normotriglyceridemic; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TC, total cholesterol; TG, triglyceride.

lipoprotein lipase or apoprotein C-II deficiency syndromes, several abnormalities have been described. For example, LDL and HDL-cholesterol levels are low, and the two are enormously enriched with TGs (13, 14). Whenever studied, both LDL and HDL are denser and smaller than the normal lipoproteins (15).

The present investigation was undertaken to determine whether abnormalities can be found in patients with milder and more common forms of HTG and whether such abnormalities, if they exist, can be ascribed to the HTG state. To this end, we have determined the nature of the plasma lipoprotein system in 16 subjects with HTG at two levels of plasma TGs, high and reduced. Bezafibrate (2-[4-[2-(4-chlorobenzamide)ethyl]phenoxy]2-methyl-propionic acid), a drug known to reduce plasma TG levels, was used to achieve the lower TG levels. This experimental design, therefore, allowed comparisons of lipoproteins in the same individual before and during TG-lowering therapy.

The study indeed revealed multiple abnormalities in all lipoprotein classes that depend on the degree of triglyceridemia.

## Methods

**Subjects.** 16 males with HTG and 7 normolipidemic male subjects agreed to participate in the study. The 16 patients were identified in the Jerusalem Lipid Research Clinic and were followed for from 6 mo to 6 yr. All patients were instructed to consume a standard isocaloric type IV diet recommended for such patients in our clinic. The diet contains 40% of calories as fats (daily cholesterol intake < 300 mg), 40% as complex carbohydrates (sugar is excluded from the diet), and 20% as protein. The patients were on this diet for at least 3 mo before they entered the study. The normal subjects consumed a regular Israeli diet, which usually was similar to that reported recently by the Jerusalem Lipid Research Clinic prevalence study (16).

The characteristics of the patients and their prestudy plasma lipid and lipoprotein-cholesterol levels are shown in Table I. Three patients

Table I. Characteristics of NTG and HTG Subjects

Patient	Age yr	Quetelet wt/ht <sup>2</sup>	Plasma		Lipoproteins			Remarks
			TG mg/100 ml plasma	Cholesterol mg/100 ml plasma	VLDL-C	LDL-C	HDL-C	
<b>HTG</b>								
1	48	28.7	764	179	90	67	22	IHD
2	66	24.2	341	178	70	84	24	IHD, F
3	55	25.0	222	196	40	132	24	IHD, F
4	44	26.2	507	240	118	94	25	IHD, F
5	48	25.4	468	220	78	114	28	DM
8	37	24.0	245	210	60	120	30	—
9	50	29.4	2,340	520	422	70	28	IHD, DM, F
10	59	25.3	890	304	189	90	25	IHD
15	40	23.6	475	220	90	100	30	IHD, F
17	44	27.6	2,500	440	380	40	20	IHD, DM
18	26	21.8	300	270	73	171	27	F
20	29	25.4	700	240	156	56	30	F
25	51	27.3	250	230	55	152	23	—
26	39	25.8	400	230	116	90	24	IHD, F
27	38	24.0	500	200	80	90	30	F
28	44	25.7	1,000	306	213	65	28	IHD
Mean±SEM	44.9±2.6	25.5±0.5	744±151	261±23	139±27	96±9	26.2±0.8	
<b>NTG</b>								
N1	31	27.1	46	123	21	69	33	
N2	48	23.5	207	174	33	108	33	
N3	38	27.4	54	178	34	107	37	
N4	41	26.2	150	199	23	143	33	
N5	33	20.4	111	202	19	146	37	
N6	33	24.6	144	214	45	137	32	
N7	45	24.3	136	231	28	161	42	
Mean±SEM	38.4±2.5	24.9±1.0	121.1±21.4	189.4±13.9	29±3.4	124.6±11.9	35.3±1.4	

IHD, ischemic heart disease; DM, diabetes mellitus; F, familial HTG.

had mild type II diabetes mellitus (fasting plasma glucose, 120 to 220 mg/dl) treated with diet only. Hepatic, renal, and thyroid functions were normal in all patients and none was receiving drug treatment for other conditions. A positive family history of HTG was obtained in nine patients. A lack of enough first-degree relatives prevented family investigations in the other patients. For the same reason, we could not classify the subjects into familial HTG or familial combined hyperlipoproteinemia. Yet all patients and their affected relatives exhibited HTG with normal or low LDL levels. Pretreatment levels of plasma TG, plasma total cholesterol (TC), very low density lipoprotein-cholesterol (VLDL-C), LDL-C, and HDL-C are shown in Table I. Similar lipid levels were also recorded on multiple occasions in all patients before they entered the study.

**Study protocol.** Patients thought to be suitable for the study were identified in the Lipid Clinic. After receiving a detailed explanation about the study and signing an informed consent form, the patients began the study. Three fasting blood samples were obtained in EDTA (1 mg/ml) before the institution of bezafibrate treatment, usually at weekly intervals. On the second visit, blood was also obtained 10 min after intravenous injection of 3,000 IU of sodium heparin (35–45 IU/kg). Plasma was promptly separated and stored at  $-20^{\circ}\text{C}$  for future (4–8 mo) determinations of heparin-releasable lipolytic activities (see below). Bezafibrate treatment, 200 mg three times per day, was instituted on the day of the third visit. On that day, a larger blood sample (60–80 ml) was obtained to determine the nature of the lipoprotein system before therapy. Blood samples (20–30 ml) were drawn after 1, 2, 4, 8, 12, 24, and 48 wk of treatment. At the end of the fourth week of treatment, a larger sample was again obtained for special studies. Also on that day, a second post-heparin sample was drawn.

Seven normolipidemic healthy males volunteered to participate in the study as controls. Some characteristics of these subjects are also shown in Table I. The procedures for obtaining plasma for lipoprotein characterization and for determining post-heparin lipolytic activities were identical to those described for the patients.

All plasma samples were drawn between 8 and 9 AM after at least a 12-h fast. The blood was kept on ice, and plasma was separated within 60 min by low speed centrifugation (3,000 rpm) for 20 min at  $4^{\circ}\text{C}$ . Post-heparin plasma samples were divided into aliquots and then immediately frozen at  $-20^{\circ}\text{C}$ . The separation of lipoproteins was also studied on the day of blood collection after the procedure detailed below.

**Preparation of lipoproteins.** Two procedures were employed to separate plasma lipoproteins. The first was identical to that of the Jerusalem Lipid Research Clinic program (17) and was used to determine VLDL, LDL, and HDL cholesterol levels. In short, plasma samples were first centrifuged at plasma density (40,000 rpm, 18 h), and the VLDL was separated by the tube slicing technique (18). Aliquots of the VLDL and of the lipoprotein fraction of  $d > 1.006$  g/ml were taken for cholesterol and TG determination. The remaining VLDL was washed once at a density of 1.006 g/ml as above and was kept for further analysis. HDL cholesterol was determined on the 1.006 g/ml infranant fraction by the heparin/manganese precipitation method, and LDL-cholesterol was calculated. The second procedure was used to obtain LDL and HDL subfractions. To that end, two 10-ml samples of the VLDL-free plasma ( $d > 1.006$  g/ml) were applied to zonal ultracentrifugation systems. The separation of LDL and HDL populations with this method was carried out as previously described (19). LDL and IDL were separated after 140 min of centrifugation in a 14 Ti zonal rotor spinning at 42,000 rpm in a 1.0–1.3 g/ml linear NaBr gradient. HDL populations were separated after 22 h of centrifugation at 41,000 rpm in a discontinuous 1.0–1.4 g/ml gradient. Eluted samples were monitored continuously at 280 nm

(UA-5 absorbance monitor; Isco, Inc., Environmental Div., Lincoln, NE) and 25 ml samples were collected. Lipoprotein fractions were identified, pooled, dialyzed against a 0.9% NaCl, 0.01%  $\text{NaN}_3$ , 0.01% EDTA, pH = 7.4 solution, and concentrated by vacuum dialysis (20). Lipoproteins were kept at  $4^{\circ}\text{C}$  for further analysis.

**Analytical procedures.** Lipoprotein protein and phospholipid were determined by standard procedures (21, 22). Total plasma cholesterol and total and lipoprotein TGs were determined by the AutoAnalyzer technique (Technicon Instrument Corp., Tarrytown, NY) (17). CE and free cholesterol content in lipoproteins were measured by the cholesterol oxidase-cholesterol esterase technique (23) with a commercial kit (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of apolipoproteins by the use of 10% gels was performed after the method described by Weber and Osborn (24). Negatively stained electron micrographs of LDL and HDL were obtained in an electron microscope (Philips 300; Philips Electronic Instruments, Inc., Mahwah, NJ) as described previously (20). Lipoproteins were photographed at 90,000 diam and prints were enlarged to 225,000 diam. For particle sizing, the diameters of 50–100 particles were measured and the mean was calculated. The standard deviation of particle distribution ranged between 5 and 10%.

Plasma apoprotein A-I (apo A-I) and plasma LDL-apoprotein B (apo B) levels were determined by immunoassays (25, 26). The apo A-I content of whole plasma was measured by a specific radioimmunoassay procedure (26). Apo B was quantified by radial immunodiffusion which used 1.2% antiserum and 1% agarose (BDH Chemicals, Ltd., Poole, England) in 0.05 M barbital buffer, pH 8.6 (25). LDL protein, determined by the Lowry procedure served as standard, and ring diameter was measured after 48 h. The standard curve was linear between 10 and 200 mg/dl. The precision and sensitivity of the immunoassay were published recently (25). All samples of an individual subject were analyzed in one assay, thus minimizing interassay variation.

**Measurements of heparin-releasable plasma lipases.** Lipoprotein lipase and hepatic TG hydrolase activities were measured in post-heparin plasma samples by two methods. Lipoprotein lipase activity was determined in samples where the hepatic TG hydrolase was completely inhibited by specific antiserum against the human enzyme. The antiserum was supplied by Dr. T. Olivecrona (University of Umea, Sweden) and was raised in rabbits against purified human hepatic TG hydrolase. The procedure used to measure lipoprotein lipase activity closely followed that described by Huttunen et al. (27). 40  $\mu\text{l}$  of post-heparin plasma was first allowed to incubate on ice with 10  $\mu\text{l}$  of immune or nonimmune rabbit serum. Thereafter, the hydrolysis of [ $^3\text{H}$ ]oleate-TGs (in washed Intralipid [Vitrum, Stockholm] [28]) was determined at  $37^{\circ}\text{C}$  in a thermostated shaking water bath. The incubation mixture (0.7 ml) contained 4 mg/ml of Intralipid, 0.05 M Tris buffer, pH 8.6, 0.1 M NaCl, and the post-heparin and control or immune rabbit plasma samples. Preliminary experiments demonstrated identical activities in mixtures with or without nonimmune rabbit plasma and complete inhibition of lipase activity in mixtures containing immune rabbit serum and 1 M NaCl. The second method used was separation of lipoprotein lipase and hepatic TG hydrolase by affinity chromatography on heparin-Sepharose CL-6B columns (Pharmacia Fine Chemicals, Piscataway, NJ) as described by Bengtsson and Olivecrona (29). Lipoprotein lipase activity was assayed in the 1.5 M NaCl eluate in the presence of 50  $\mu\text{l}/\text{ml}$  of normal human serum, and hepatic TG hydrolase activity was assayed in the 0.7 M eluate in the absence of serum. Hepatic TG hydrolase activity was calculated from the total post-heparin lipolytic activity, the ratios of heparin-Sepharose eluted activities, and the directly measured lipoprotein lipase

activity. Post-heparin plasma samples obtained from the same patient before and 4 wk after the initiation of therapy were always included in the same assay.

**Statistical analysis.** Tests for significant differences between untreated and treated HTG patients were performed by paired *t* test evaluation and between NTG subjects and untreated HTG patients by unpaired *t* test evaluation. Linear regression analysis was used to determine coefficients of correlation between lipoprotein parameters and lipase activities as compared with plasma TG levels (log transformed). Significance levels were obtained by *t* and *r* tables (30).

## Results

**Plasma lipids and lipoprotein-lipid levels.** Mean plasma TG decreased by  $56 \pm 4.3\%$  (mean  $\pm$  SEM,  $P < 0.005$ ) after 1 wk of treatment and remained at that low level for the 48 wk of study (Fig. 1 *a*). The decrease of plasma TG was observed in all subjects and ranged between 29 and 82% (Fig. 1 *b*). A moderate decrease of mean plasma cholesterol from  $257 \pm 21$  to  $220 \pm 9.3$  mg/dl ( $P < 0.05$ ) was observed after 1 wk and to  $214 \pm 6.8$  mg/dl ( $P < 0.05$ ) after 2 wk (Fig. 2). At subsequent examinations, mean plasma cholesterol increased but remained lower than pretreatment levels. The change of plasma cholesterol in individual subjects ranged from  $+18.5\%$  to  $-51.6\%$ , and the maximal decrease was seen in subjects with the highest plasma TG

levels (patients 9, 10, 17, and 28). Lipoprotein-C levels are also shown in Fig. 2. The change of VLDL-C paralleled that of plasma TG, and a mean decrease from  $144 \pm 30$  to  $60 \pm 14$  mg/dl was observed after 1 wk of treatment ( $P < 0.005$ ). LDL-C and HDL-C levels increased in all patients. After 1 wk of treatment, mean LDL-C increased from  $96 \pm 8.6$  to  $131 \pm 8.5$  mg/dl ( $P < 0.005$ ). The highest LDL-C was observed after 4 wk, at  $139 \pm 7.4$  mg/dl. HDL-C increased from  $26.2 \pm 0.8$  to  $32.9 \pm 1.2$  mg/dl ( $P < 0.005$ ) after 1 wk and peaked at 12 wk at  $36.3 \pm 2.0$  mg/dl. Thus, the distribution of cholesterol among lipoproteins changed markedly with treatment from very abnormal distribution towards that found in the NTG subjects (Table II).

**VLDL.** VLDL was isolated and characterized before and 4 wk after treatment was started (Table III). Several abnormalities of HTG-VLDL are evident: low protein and TG content and high CE and free cholesterol content. The CE-to-protein and CE-to-TG mass ratios in HTG-VLDL are twice those in *N*-VLDL, and the CE to phospholipid ratio is  $\sim 50\%$  above normal. After 4 wk of treatment, VLDL composition changed towards that observed in normal subjects. Linear regression analysis of the compositional data against plasma TG levels was performed separately for untreated subjects (NTGs and HTGs) and for all subjects, and included values measured after 4 wk of treatment (Table IV). Highly significant negative relationships

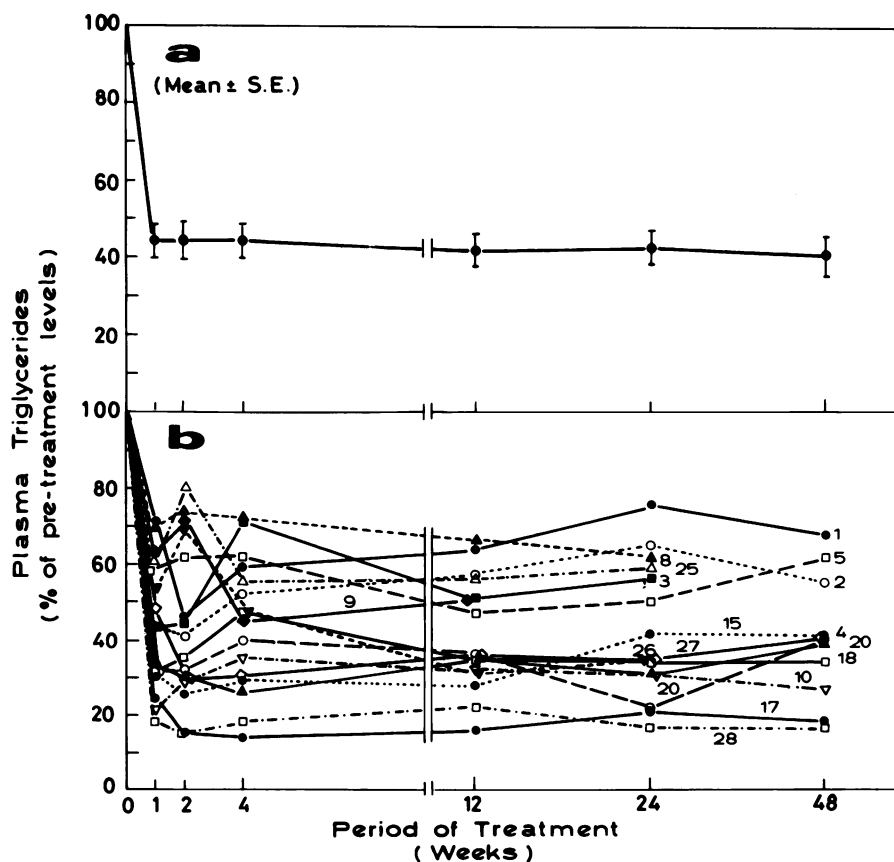


Figure 1. Relative plasma TG levels in HTG subjects before and during treatment with bezafibrate. (a) Mean  $\pm$  SEM value for the 16 subjects; (b) individual values.

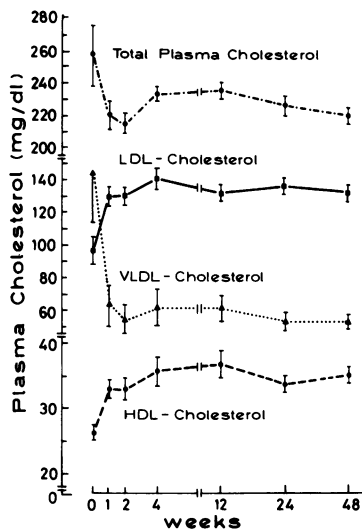


Figure 2. TC content in the plasma and the individual lipoproteins before and during bezafibrate treatment. Values are mean±SEM for the 16 HTG subjects.

were found; relationships were negative for VLDL-protein and positive for the CE-to-protein ratio and for the VLDL-free cholesterol content. The relationships for VLDL-TG (negative) and VLDL-CE (positive) were also significant ( $P < 0.05$ ). It is interesting that when the two subjects with plasma TG levels above 1,000 mg/dl were deleted from the analysis, the coefficient of correlation between VLDL-CE and plasma TG rose to 0.67 ( $P < 0.001$ ). Fig. 3 illustrates the distribution of individual CE-to-protein ratios against plasma TG levels. In spite of variation of the ratio at any plasma TG level, the two are highly correlated ( $r = 0.76$  and  $0.65$ ,  $P < 0.001$ ). In general, coefficients of correlation become lower when values obtained after 4 wk of treatment are included in the analysis, but the parameters of the regressions (intercepts and slopes) do not change much.

VLDL-apoprotein profiles were studied by SDS-PAGE. Relative amounts of apo B and apoprotein C did not differ much between subjects and were not affected by treatment. Relative amounts of apoprotein E differed between subjects, and with bezafibrate treatment changed inconsistently. Other apoproteins, including apo A-I, were not detected.

**LDLs.** LDL was analyzed by zonal ultracentrifugation of VLDL-free plasma ( $d > 1.006$  g/ml) before and 4 wk after 14 patients started treatment (Fig. 4). In normal subjects, two fractions were observed. The lighter fraction was present in relatively low amounts and could be eliminated when IDL ( $d$ , 1.006–

1.019 g/ml) was removed. The main fraction eluted with a peak at 215–245 ml of the zonal rotor volume and represented the true LDL. In HTG, abnormal elution profiles were observed. These profiles could be classified into at least three categories. Category one was observed in four patients and was characterized by an elevation of the lighter fraction (IDL, 75–175 ml of elution volume) and a slight shift of density of the main LDL fraction (Fig. 4 b). Five patients were included in category two. Their LDL was characterized by the presence of a third fraction between IDL and LDL and a further shift of density of the main LDL (Fig. 4 c). In the third category (five patients), the LDL exhibited multiple abnormal fractions (Fig. 4, d and e), and the peak we defined as main LDL eluted at zonal volumes as late as 320 ml. Mean plasma TG levels of the three groups of patients increased progressively and were  $296 \pm 58$ ,  $436 \pm 40$ , and  $1,486 \pm 391$  mg/dl, respectively. Bezafibrate treatment caused a change of LDL profiles according to the decrease of plasma TG (Fig. 4, broken lines). Two further analyses were performed with the main LDL data obtained directly from the zonal ultracentrifuge: linear regression analysis of the peak elution volume against plasma TG, and the calculation of the area of the main LDL (in arbitrary units of  $\text{cm}^2$ ). The peak elution volume (which estimates the density of the lipoprotein) was significantly correlated with plasma TG (Table IV, Fig. 5 A). The area of the main LDL fraction was  $3.89 \pm 0.82$   $\text{cm}^2$  (mean±SE) and that value increased to  $7.06 \pm 0.82$  after 4 wk of treatment ( $P < 0.0001$ ). The LDL area was inversely correlated with the plasma TG levels (Table IV).

The chemical composition of the main LDL fraction is shown in Table III, the values of the linear regression analysis in Table IV, and the relationships between CE-to-protein and CE-to-TG ratios against plasma TG levels in Fig. 5 B and C. HTG-LDL was characterized by high protein and TG content, low CE and free cholesterol content, and low CE-to-protein and CE-to-TG ratios. These deviations from normal LDL were all highly significant and strongly and significantly related to plasma TG. The contribution of total phospholipids to HTG-LDL also decreased with the degree of triglyceridemia. Bezafibrate treatment caused a reversal towards normal of all measured parameters. As shown in Table IV, the addition of values obtained during treatment affected mainly the coefficient of correlations but not the regression curve, i.e., the intercept and slope.

Negatively stained electron micrographs of the main LDL were available in 13 patients and 6 normal subjects. Fig. 6

Table II. Effect of Bezafibrate Treatment on the Relative Distribution of Cholesterol in the Various Lipoproteins

	Normal subjects	HTG patients (weeks of treatment)				
		0	1	4	12	48
VLDL-C/TC	0.15±0.02	0.51±0.05*	0.27±0.04	0.26±0.04	0.26±0.03	0.24±0.02
LDL-C/TC	0.65±0.03	0.41±0.05*	0.60±0.04	0.61±0.03	0.57±0.03	0.61±0.02
HDL-C/TC	0.19±0.02	0.11±0.01‡	0.15±0.01	0.16±0.01	0.16±0.01	0.16±0.01

Values are mean±SEM of 7 normolipidemic and 16 HTG subjects. \*  $P < 0.001$ , ‡  $P < 0.01$ ; both by unpaired  $t$  test.

Table III. Relative Composition of Lipoproteins in NTG and HTG Subjects Before (Untreated) and During (Treated) Bezafibrate Treatment

Lipo-protein	Subjects	Protein	TG	CE	FC	PL	CE/PR	CE/PL	CE/TG
VLDL	HTG, untreated	10.21±0.52	53.42±1.73	11.71±0.26	6.23±0.3	18.41±0.74	1.184±0.09	0.64±0.04	0.23±0.02
	HTG, treated	12.27±0.62*	55.9±1.4*	8.79±0.6*	4.94±0.24*	18.22±0.69	0.75±0.07*	0.48±0.03‡	0.16±0.01*
	NTG	14.2±0.46§	55.8±0.92	8.3±0.65§	4.2±0.36§	17.5±0.8	0.58±0.06§	0.44±0.05 <sup>  </sup>	0.126±0.01§
LDL	HTG, untreated	27.79±1.18	10.59±1.64	34.69±1.7	6.71±0.53	20.16±0.51	1.31±0.11‡	1.72±0.07	4.06±0.63
	HTG, treated	22.92±0.94‡	7.38±0.77‡	39.55±1.23*	8.95±0.36*	22.42±0.45*	1.77±0.1*	1.77±0.05*	6.01±0.72*
	NTG	21.7±0.6§	5.9±1.08	41.4±0.64§	9.2±0.47 <sup>¶</sup>	21.7±0.64§	1.97±0.169 <sup>¶</sup>	1.91±0.2	7.34±1.2§
HDL	HTG, untreated	53.2±1.2	8.54±0.92	12.4±0.57	1.88±0.08	23.91±1.23	0.23±0.01	0.54±0.05	1.73±0.23
	HTG, treated	52.0±0.95	5.22±0.66**	14.58±0.67*	1.86±0.1	26.28±0.54‡	0.28±0.01*	0.56±0.03	3.16±0.59‡
	NTG	52.0±1.7	4.68±0.46§	18.52±0.49§	2.07±0.11	22.75±1.64	0.36±0.01§	0.83±0.08 <sup>¶</sup>	4.16±0.47§

Values are expressed in weight percent or weight ratios (mean±SEM). \*  $P < 0.001$ , ‡ $P < 0.01$ , \*\* $P < 0.05$ ; all paired  $t$  test, treated vs. untreated HTG subjects. §  $P < 0.001$ , ¶ $P < 0.05$ , <sup>||</sup> $P < 0.05$ ; all unpaired  $t$  test, normolipemics vs. untreated HTG subjects.

presents electron micrographs from one normal subject and three patients with HTG; each represents a patient of one of the three different categories. HTG-LDL is smaller than normal LDL, and LDL size increases with therapy. The smallest LDL was found in patient 17, with diameter of 167 Å (normal 200–220 Å). As for other LDL parameters, the diameter of the LDL was strongly and significantly related (negatively) to plasma TG (Fig. 5 D, Table IV).

SDS-PAGE of the main LDL revealed that apo B was the main apoprotein present. It is estimated that more than 90% of the total protein content was accounted for by apo B. Small amounts of apoprotein C were also identified. The apoprotein profile of LDL was not changed by treatment. Apo B content of VLDL-free plasma ( $d > 1.006$  g/ml) in the 16 patients before the initiation of bezafibrate treatment was 95±4.5 mg/dl (mean±SEM). This value increased to 110±4.4 mg/dl after 4 wk of treatment ( $P < 0.05$ ). With treatment, the cholesterol-to-apo B weight ratio also increased, from 1.02 to 1.33 ( $P < 0.005$ ). A more pronounced increase was found for the main LDL protein and CE content, from 38±4.1 to 55±6.6 mg/dl ( $P < 0.05$ ) and from 54±8.8 to 93±13.6 mg/dl ( $P < 0.05$ ), respectively.

**HDLs.** HDL populations were isolated from the plasma of 13 patients and 7 normal subjects. In HTG, the predominant HDL population was HDL<sub>3</sub> (Fig. 4). HDL<sub>2</sub> was either barely identified or not present at all. The analysis therefore is confined to HDL<sub>3</sub>.

HTG-HDL<sub>3</sub> eluted in the zonal rotor later than *N*-HDL<sub>3</sub>

(peak elution volumes of 285±4.8 and 260±7.0 ml (mean±SEM), respectively). Peak elution volumes were significantly related to plasma TG levels (Fig. 7 A and Table IV). Bezafibrate treatment caused a shift of HDL<sub>3</sub> towards a lower density in 10 of the 13 patients (examples in Fig. 4), with a peak elution volume of 271±6.1 ml (mean±SEM,  $P < 0.01$ , as compared with pretreatment volumes).

The chemical composition of HDL<sub>3</sub> is shown in Table III and the linear regression analysis of the relationships of HDL<sub>3</sub> constituents to plasma TG is shown in Table IV and Fig. 7 B and C. HTG-HDL<sub>3</sub> was characterized by a minor elevation of protein content, an increased TG and decreased CE content, and low CE-to-protein, CE-to-TG, and CE-to-phospholipid ratios. Except for the protein, all other parameters were significantly related to plasma TG levels (Table IV). Bezafibrate treatment caused all abnormalities to reverse toward normal. SDS-PAGE demonstrated that apo A-I and apoprotein A-II were the predominant HDL<sub>3</sub> apoproteins, and no qualitative differences were noted between groups of subjects.

Plasma apo A-I was measured by radioimmunoassay during the first 12 wk of treatment in eight patients (Fig. 8). With treatment, plasma HDL-C in these eight patients increased by 33.7%, whereas plasma apo A-I content increased by only 5.1%. Thus, the ratio of plasma apo A-I to HDL-C decreased by 22.0%.

**Heparin-releasable plasma lipases.** The activity of the heparin-releasable plasma lipoprotein lipase and the hepatic TG hydrolase are shown in Table V and Fig. 9. Both activities were low in HTG before treatment. A highly significant negative

Table IV. Correlation between Lipoprotein Parameters and Plasma TG Levels

Lipoprotein	Parameter	NTG and untreated HTG subjects			NTG and untreated and treated HTG subjects		
		Intercept	Slope	r	Intercept	Slope	r
VLDL	Protein	27.52	-6.34	-0.95*	21.95	-4.17	-0.56*
	PL	18.16	-0.08	-0.01	18.64	-0.25	-0.04
	CE	4.54	2.54	0.38‡	2.98	2.93	0.40‡
	TG	63.95	-4.45	-0.43	66.02	-4.84	0.36‡
	FC	-0.78	2.52	0.79*	-0.89	2.54	0.78*
	CE/PR	-0.83	0.74	0.76*	-0.76	0.69	0.65*
LDL	Protein	5.84	7.86	0.78*	5.34	7.83	0.71*
	PL	24.61	-1.70	-0.47‡	27.20	-2.40	-0.49‡
	CE	65.53	-11.12	-0.80*	62.6	-9.89	-0.68*
	TG	-4.65	5.06	0.46‡	-5.25	5.33	0.50§
	FC	16.34	-3.50	-0.78	17.26	-3.74	-0.77
	CE/PR	3.64	-0.83	-0.82*	3.61	-0.81	-0.73*
	CE/TG	16.04	-4.30	-0.78*	16.50	-4.47	-0.69*
	Peak	129	51.2	0.94*	113	54.4	0.82*
	Area	18.03	-5.13	-0.56§	21.79	-6.44	-0.70*
Diameter	276.9	-31.8	-0.78§	284.5	-33.67	-0.62*	
HDL <sub>3</sub>	Protein	42.57	3.12	0.13	48.0	1.18	0.05
	PL	20.09	1.47	0.18	22.0	1.16	0.14
	CE	27.77	-6.08	-0.83*	28.71	-5.78	-0.77*
	TG	-0.81	3.10	0.51‡	-3.81	3.84	0.58*
	FC	2.25	-0.12	-0.20	2.27	-0.14	-0.19
	CE/PR	0.57	-0.12	-0.74*	0.54	-0.11	-0.66*
	CE/TG	9.40	-2.70	-0.85*	10.03	-2.91	-0.69*
	Peak	205.9	28.40	0.71*	202.7	28.40	0.44§
Lipoprotein lipase	18.8	-5.35	-0.74*	22.0	-6.35	-0.89*	
HTGL	57.3	-17.86	-0.76*	47.8	-14.61	-0.66*	

Data are results of linear regression analysis of lipoprotein composition (in weight percent), peak elution volume and area (of main LDL or of HDL<sub>3</sub>), LDL diameter, and plasma lipases activity as related to plasma log TG levels. \*  $P < 0.001$ ; §  $P < 0.01$ . ‡  $P < 0.05$ . PL, phospholipids; FC, free cholesterol; Peak, peak elution volume in the zonal centrifuge area; HTGL, hepatic triglyceride hydrolase.

relationship was found between each activity and plasma TG levels (Table IV). Bezafibrate treatment caused an increase of lipoprotein lipase activity in all subjects (Fig. 9), and the mean activity in the treated subjects approached that of the normal subjects. With treatment the hepatic TG hydrolase activity increased by more than 30% in 12 patients but on the average was considerably lower than in normal subjects. During bezafibrate treatment, the contribution of lipoprotein lipase to total heparin-releasable lipolytic activity was therefore the highest—48.1% as compared with 25.3% in normals and 36.6% in untreated HTG.

### Discussion

The present investigation was undertaken to elucidate the nature of the lipoprotein system in HTG in two states: high and reduced

plasma TG levels. Our working hypothesis was that HTG states have profound effects on all plasma lipoproteins and that these effects, in part or on the whole, reflect the degree of triglyceridemia. We further hoped that elucidation of abnormalities in HTG lipoproteins would prove relevant to physiological and pathophysiological metabolic events that are associated with HTG. Bezafibrate, a derivative of fibric acid, was chosen as a TG-lowering drug (31), and the plasma lipoproteins were rigorously characterized in 16 HTG subjects before and 4 wk after the initiation of treatment. Bezafibrate has been reported to increase the activity of heparin-releasable lipases (32), an effect also demonstrated in the present study.

Abnormalities related to the degree of triglyceridemia were found in all major lipoproteins, and these abnormalities tended to reverse towards normal when plasma TGs were reduced. The observation that with treatment the regression parameters for

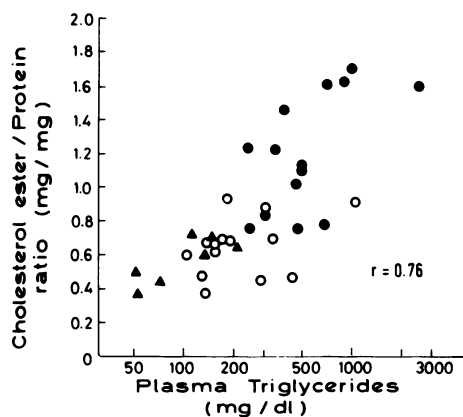


Figure 3. The distribution of individual VLDL-CE-to-protein ratios against plasma TG levels.  $\blacktriangle$ , normal subjects;  $\bullet$ , untreated HTG subjects;  $\circ$ , HTG subjects after 4 wk of bezafibrate treatment. The  $r$  value is for the HTG untreated and normal groups.

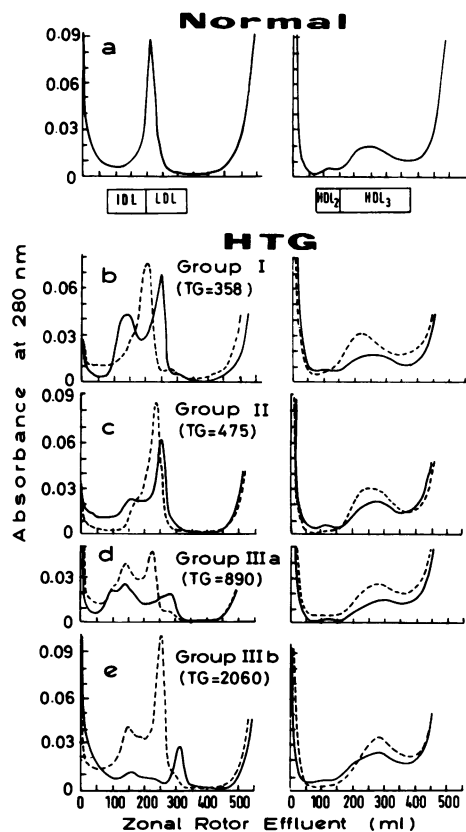


Figure 4. Zonal ultracentrifugate elution profiles of lipoproteins from normal and HTG subjects (—) and from HTG subjects after 4 wk of bezafibrate treatment (---). In the left column are LDL elution profiles; in the right column are HDL elution profiles (see Methods). Elution profiles of HTG subjects (b–e) represent the categories described in Results, and a presents the profile of a normal subject. The range of the elution volume can be 75–175 ml of the effluent volume for IDL, 175–350 ml for LDL, 75–150 ml for HDL<sub>2</sub>, and 150–375 ml for HDL<sub>3</sub>, as indicated in a.

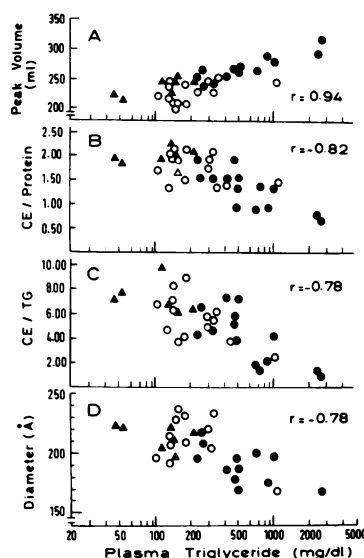


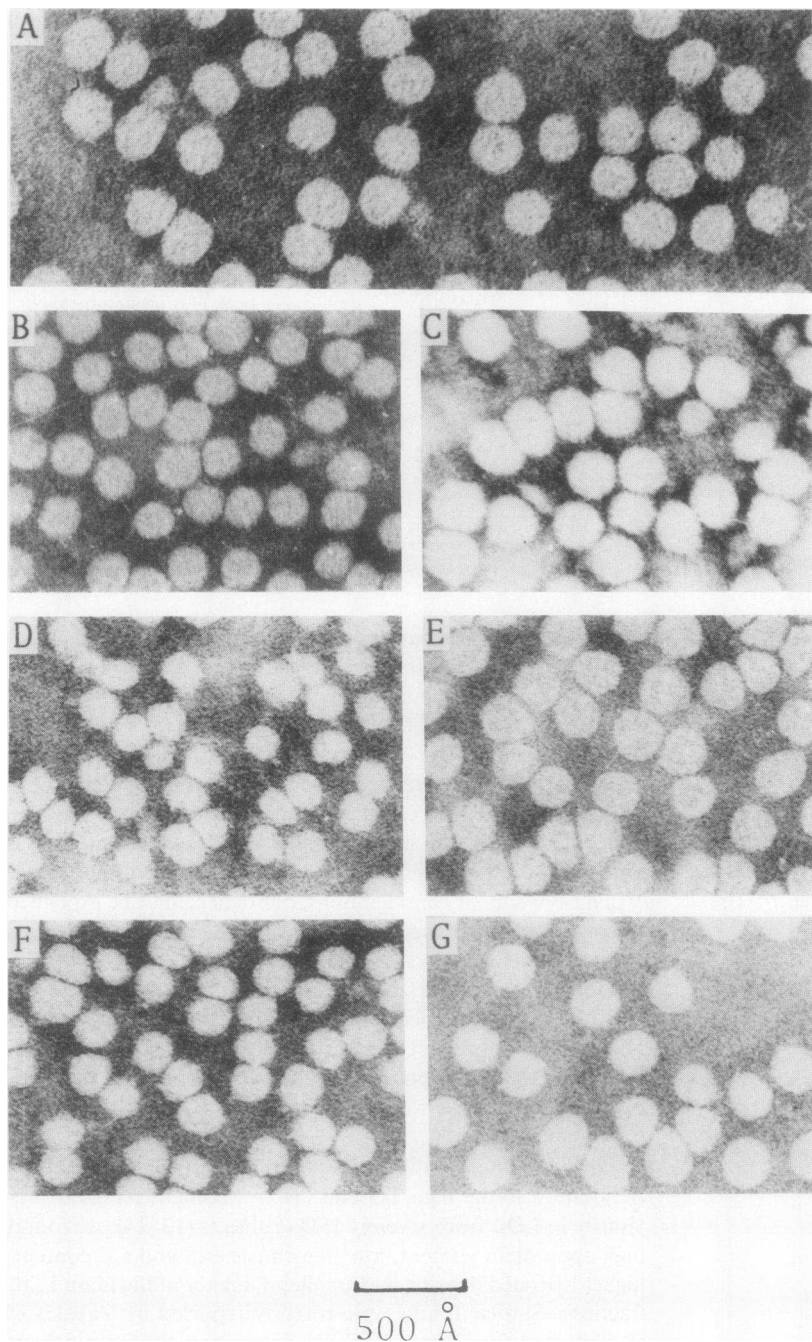
Figure 5. The relationship of individual LDL parameters to plasma TG levels. Parameters are: (A) zonal peak elution volumes; (B) CE-to-protein weight ratios; (C) CE-to-TG weight ratios; and (D) diameters of the main LDLs.  $\blacktriangle$ , normal subjects;  $\bullet$ , untreated HTG subjects;  $\circ$ , bezafibrate-treated subjects. The  $r$  values are for the HTG untreated and normal groups.

most measured data have not changed much supports the concept that most abnormalities indeed reflect the degree of triglycerides or the basic metabolic pathways that are responsible for the HTG state. HTG-VLDL is depleted of apoproteins and TGs and is enriched in CEs and free cholesterol. While the low protein content may reflect a predominance of lighter VLDL populations (1, 33–36), this possibility fails to explain the lipid abnormalities. In fact, light and large VLDL contain more TGs and less cholesterol (free and ester) than does small and dense VLDL. For similar considerations, our data cannot be explained by contamination of the VLDL with chylomicrons; chylomicrons contain more TGs and less cholesterol than does VLDL (37). We therefore conclude that the abnormal lipid composition of HTG-VLDL must reflect a metabolic defect related to the state of HTG and not merely a changing subpopulation distribution. This conclusion is supported by the observation that these abnormalities diminish with the reduction of HTG by bezafibrate treatment.

Several abnormalities were identified in the LDL fraction. In addition to the high TG and low TC content reported previously in LDL from severely HTG subjects (13, 14), we found high apoprotein content, low free cholesterol and CE content, higher hydrated density, and smaller diameter of the main LDL fractions. Similar results were recently reported by Vakakis et al. while studying a group of HTG subjects from the Framingham cohort (38). In the present investigation, we show that all these abnormalities are strongly and significantly related to the degree of triglyceridemia and are potentially reversible when plasma TG levels are reduced. Strong relationships between plasma TG levels and TC-to-TG ratios in LDL (39) and HDL (39, 40) have also been described in populations where the majority of the subjects are NTGs. Free and esterified cholesterol, however, were not separated in these studies.

Bezafibrate treatment caused an increase in all LDL constituents except TGs. Similar observations were reported during





*Figure 6.* Electron micrographs of normal LDL and main peak HTG-LDLs before and after 4 wk of bezafibrate treatment. Normal LDL (A): main LDL of a group I subject before (B) and during (C) bezafibrate treatment. Main LDL of a group II subject before (D) and during (E) bezafibrate treatment. Main LDL of a group III subject before (F) and during (G) bezafibrate treatment.

treatment of HTG patients with clofibrate (41, 42). The increase of LDL protein and apo B levels indicates an increased number of LDL particles. Bezafibrate has been reported to cause an increased LDL-receptor activity and to decrease LDL in patients with normal plasma TG levels (e.g., type IIA hyperlipoproteinemia) (43). This effect of bezafibrate is expected to reduce HTG-LDL levels, contrary to our findings. It therefore appears that bezafibrate also increases LDL production, which in physiologic

terms reflects increased VLDL-to-LDL conversion (1, 2). Incomplete VLDL-to-LDL conversion in HTG (of any type) has been reported in almost all VLDL-apo B turnover studies conducted in such patients (44–48). Bezafibrate treatment apparently corrects this defect, at least in part. This phenomenon may also reflect the state of HTG and the abnormal distribution of core-lipids between TG-rich lipoproteins and CE-rich lipoproteins (discussed below). It is proposed that in HTG, VLDL particles

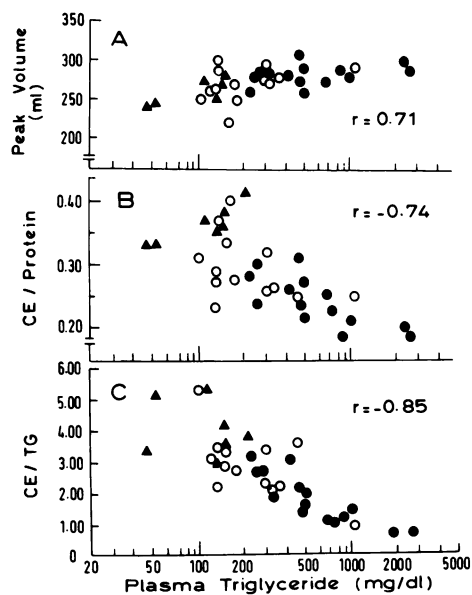


Figure 7. The relationship of individual HDL parameters to plasma TG levels. Parameters are (A) zonal peak elution volumes; (B) CE-to-protein weight ratios; and (C) CE-to-TG weight ratios. ▲, normal subjects; ●, untreated HTG subjects; ○, bezafibrate-treated subjects. The  $r$  values are for the HTG untreated and normal groups.

contain surplus CE molecules and therefore cannot complete the VLDL → IDL → LDL conversion process. As we described in an abstract, this indeed is the case for most, if not all, large and light HTG-VLDL populations (49).

The abnormalities found in the HDL system are similar to those of LDL, i.e., the HDL belongs predominantly to the small, heavier HDL<sub>3</sub> subpopulation and is TG and protein rich and CE poor. Dense HDL populations have previously been described in HTG (50). The present study demonstrates that the appearance of such HDL populations depends at least in part

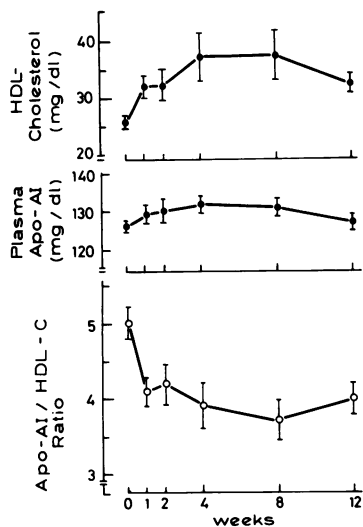


Figure 8. The effect of bezafibrate on HDL cholesterol, plasma apo A-I, and the apo A-I/HDL-C ratio during 12 wk of bezafibrate treatment. Values indicate the mean and SEM variations.

Table V. Effect of Bezafibrate Treatment on Heparin-Releasable Plasma Lipases

	LPL	LPL	HTGL
	% of PHLA	$\mu\text{mol FA/ml per h}$	$\mu\text{mol FA/ml per h}$
HTG, untreated	$36.6 \pm 3.7$	$3.4 \pm 0.57$	$4.6 \pm 1.02$
HTG, treated	$48.1 \pm 4.5$	$8.9 \pm 1.37^*$	$9.8 \pm 1.41^*$
NTG	$26.3 \pm 3.5$	$8.8 \pm 0.84^*$	$25.2 \pm 3.32^*$

Data are mean  $\pm$  SEM of 16 HTG patients before (untreated) and 4 wk after initiation of bezafibrate treatment (treated), and of 7 NTG subjects. LPL, lipoprotein lipase; PHLA, post-heparin plasma Lipolytic activity; HTGL, hepatic triglyceride hydrolase, FA, fatty acids. \*  $P < 0.001$ , unpaired  $t$  test.

on the degree of triglyceridemia and that it is partially reversible when plasma TG levels are reduced. Bezafibrate caused an increase of HDL mass and HDL-C levels with only a minimal change of plasma apo A-I levels. This probably reflects the increased lipoprotein lipase activity caused by bezafibrate. Numerous studies have demonstrated that HDL-C levels are directly related to the activity of the lipoprotein lipase system (4, 5), owing to the higher transfer of surface remnants from lipolyzed TG-rich lipoproteins to HDL (6, 51). The effects of the lipase on apo A-I are presumably less dominant.

Our data indicate that decreased plasma lipase activity is an important factor that determines the presence and degree of HTG. This conclusion agrees with previous reports (52, 53) and with the concept that in humans TG production rates vary and the degree of triglyceridemia depends on the removal mechanisms (54, 55). Decreased lipase activity explains some of our

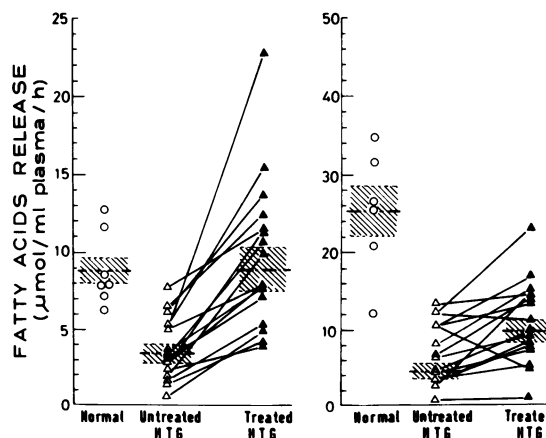


Figure 9. The activities of heparin-releasable lipoprotein lipase (left) and hepatic TG lipase (right) in normal subjects (○), untreated HTG subjects (△), and HTG subjects after 4 wk of bezafibrate treatment (▲). Dashed line (---) and shaded areas indicate the mean and SEM variations.

observations, for example the high VLDL levels, the high TG levels in LDL and HDL and their reduction with treatment, and the increased lipase activity. Yet all other observations cannot be directly related to the lipase system, although they are strongly and linearly related to the plasma TG levels. Therefore, in HTG, another metabolic activity must be present to account for high CE (and free cholesterol) content in VLDL, low CE content in LDL and HDL, very high CE/protein ratios in VLDL, very low ratios in LDL and HDL, and for the presence of both LDL and HDL as abnormally dense and small populations. A mechanism that may explain some of these abnormalities is the activity of plasma core lipid transfer proteins. These proteins are known to initiate a transfer of CEs from LDL and HDL to chylomicrons and VLDL, and of TGs in the opposite direction (12, 56–59). Such transfers are readily demonstrated in vitro and are related to the mass ratio between VLDL and LDL or HDL and to the duration of the incubation (56–59) and may be even more pronounced in vivo. Thus, the presence of a large and slowly metabolized HTG-VLDL mass should entail an excessive transfer of CE to VLDL and of TG to LDL and HDL. This agrees well with our observation that the core-lipid compositional changes are highly correlated with plasma TG levels. Furthermore, the increased density and decreased size of LDL and HDL can also be related to the core-lipid transfer reaction. We suggest that transferred TGs can be hydrolyzed by plasma lipases, yielding populations that gradually become smaller and heavier than normal. It has been demonstrated that LDL TGs, either transferred in vitro (56) or in LDL isolated from plasma (60), are slowly hydrolyzed by both lipoprotein lipase and hepatic lipase preparations. Bezafibrate, by enhancing lipoprotein lipase, speeds the metabolism of VLDL and reduces its plasma mass. This results in decreased core-lipid transfer and the appearance of normalized lipoprotein populations. Moreover, with the reduction of plasma TG levels, fewer CEs are transferred to VLDL and more particles can be successfully converted to LDL via the VLDL → IDL → LDL cascade. Although the pathway discussed above explains most of the abnormalities found in HTG-LDL, it fails to explain the presence of multiple LDL fractions and the low free-cholesterol content of the lipoprotein. The increased free cholesterol content of HTG-VLDL also remains unexplained.

The abnormalities of the lipoprotein system discussed above have important metabolic implications and are potentially atherogenic. The main metabolic consequence is related to the abnormal distribution of cholesterol among lipoprotein classes. Regression analysis demonstrates that even with moderate triglyceridemia (e.g., TG levels of 500 mg/dl), LDL particles contain only 50% of the amount of cholesterol (predominantly esterified, but also free) present in *N*-LDL, with TG levels of 100 mg/dl (footnote 2). Upon catabolism, the amount of cholesterol that

leaves the plasma with such HTG-LDL particles is also one half that degraded with NTG-LDL. The degradation of HTG-LDL particles, therefore, will cause less down-regulation of the LDL-receptor and some increased catabolism of LDL, as reported in HTG (46, 61). In spite of this increased fractional catabolic rate of LDL, it is predicted that the exit of cholesterol from the plasma with this lipoprotein is substantially lower than in NTG. (If cholesterol degradation with LDL becomes normalized, LDL-receptor activity will also be decreased, and the accelerated LDL catabolism will be inhibited.) Similar considerations lead to the conclusion that the exit of cholesterol from plasma with HDL is also decreased in HTG subjects. A great deal of cholesterol must then be cleared from the patients' plasma through pathways that are unrelated to either LDL or HDL catabolism. It is suggested that this cholesterol exits with the CE- and free-cholesterol-rich VLDL particles. Because such particles are unable to complete the VLDL-to-LDL conversion pathway, they must be degraded directly, in the form of VLDL or VLDL remnants. The clearance of cholesterol from plasma via the LDL receptor is currently thought to provide protection against atherosclerotic process (62). The exit of cholesterol from plasma with VLDL or IDL through other pathways, as described recently by Gian-turko et al. (63) is, in contrast, potentially atherogenic.

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plasma TG levels (276.9–31.8 log TG), is to 64%. When both effects are combined, it is calculated that the amount of CEs in the CE-poor, small HTG-LDL at plasma TG levels of 500 mg/dl is 46% that at 100 mg/dl.

2. The regression parameters shown in Table IV allow calculations of the amounts of CE in LDL particles. The equation for CE/protein (3.63–0.83 log TG) predicts a weight ratio of 1.98 at plasma TG levels of 100 mg/dl, and of 1.40 at 500 mg/dl, or a decrease to 71%. The decrease in core volume, derived from the relationship between diameter and

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