Measurement of two plasma triglyceride lipases by an immunochemical method: studies in patients with hypertriglyceridemia

H. Greten, R. DeGrella, G. Klose, W. Rascher, J. L. de Gennes, and E. Gjone

Klinisches Institut für Herzinfarktforschung, Medizinische Universitätsklinik Heidelberg, 69 Heidelberg, Germany

Abstract Postheparin plasma lipolytic activity consists of two hydrolytic activities, hepatic triglyceride lipase and lipoprotein lipase. These two enzymes were separated and partially purified by means of ammonium sulfate precipitation and affinity chromatography using Sepharose with covalently linked heparin and concanavalin A, respectively. Antibodies were produced against hepatic triglyceride lipase and they did not cross react with lipoprotein lipase. Optimal conditions for selective precipitation of hepatic lipase and specific measurement of these two lipases were investigated. This method was applied to the study of 15 patients with hypertriglyceridemia and 8 patients with familial lecithin-cholesterol-acyltransferase deficiency of whom 6 also had a marked elevated plasma triglyceride concentration. All patients had normal values of hepatic plasma lipase. All 8 patients with Type I and 2 of 4 patients with Type V hyperlipoproteinemia had lipoprotein lipase activities that were markedly reduced. The patients with Type III hyperlipoproteinemia and all 8 patients with lecithin-cholesterol-acyltransferase deficiency also had normal lipoprotein lipase values. These studies emphasize the necessity for differentiating between triglyceride lipase activity of hepatic and extrahepatic origin in evaluating patients with impaired triglyceride metabolism.

Supplementary key words Postheparin lipolytic activity · lipoprotein lipase · hepatic triglyceride lipase · enzyme antibodies · purification · hyperlipoproteinemia · lecithin-cholesterol-acyltransferase deficiency

Postheparin plasma lipolytic activity (PHLA) consists of two hydrolytic activities, hepatic triglyceride lipase (H-TGL) and lipoprotein lipase (LPL) originating respectively from liver and extrahepatic tissues (1–7). These two enzymatic activities can be separated by affinity chromatography on Sepharose with covalently linked heparin and can be shown to possess different characteristics (8). LPL requires an apolipoprotein cofactor for full activity and is inhibited when assayed in the presence of protamine sulfate or high salt. H-TGL does not require a cofactor, it is not inhibited by protamine sulfate and it is maximally active when assayed in solutions containing 0.5–1.0 M NaCl (9).

Intravascular catabolism of circulating lipoprotein triglyceride is believed to be mediated by these two lipases, even though the exact mode of action has not been demonstrated. Hypertriglyceridemia in several metabolic disorders has been shown to be associated with deficiency of post-heparin lipolytic activity. Recent reports have shown that selective measurement of the two triglyceride lipases can be performed on the basis of inactivation of LPL by protamine sulfate (6) or immunochemical methods (10, 11).

The reported separation and partial purification of H-TGL and LPL were modified and extended. Antibodies were produced against H-TGL that did not cross react with LPL. Optimal conditions for the selective precipitation of H-TGL and specific measurement of these two lipases were investigated. This assay was employed in the measurement of H-TGL and LPL in patients with various forms of impaired lipoprotein metabolism. Of special interest were patients with Type I, Type III, and Type V hyperlipoproteinemia and patients with familial lecithin-cholesterol-acyltransferase (LCAT) deficiency. The latter have an abnormally large low density lipoprotein that is thought to be derived from triglyceride-rich lipoproteins, suggesting a possible defect in triglyceride lipase activity (12).

METHODS

Enzyme source

Unless otherwise stated all blood samples were drawn 10 min after intravenous injection of heparin,

Abbreviations: PHLA, postheparin lipolytic activity; H-TGL, hepatic triglyceride lipase; LPL, lipoprotein lipase; LCAT, lecithin-cholesterol-acyltransferase; BSA, bovine serum albumin; LDL, low density lipoproteins.

TABLE 1. Laboratory findings in patients with hypertriglyceridemia

Patient	Age	Sex	Plasma Triglyceride	Plasma Cholesterol	Lipoprotein Electrophoresis Pattern	H-TGL	LPL	Oral Glucose Tolerance Test
			mg/100 ml	*mg/100 ml*		*μmoles FFA/ml/hr*	*μmoles FFA/ml/hr*	
U.K.	25	F	7800	1260	I	0.32	0.08	normal
W.R.	23	M	6725	780	I	2.36	0.27	normal
W.C.	9	M	4000	300	I	2.63	0.09	normal
P.V.	9	M	6400	900	I	3.67	0.12	normal
J.B.	14	M	1520	330	I	8.47	0.71	normal
C.M.	17	F	2000	450	I	4.12	0.40	normal
C.A.	2	F	4200	450	I	3.06	0.04	normal
Mc.M.	9	F	1180	660	I	4.32	0.29	
A.K.	69	M	380	348	III	11.00	5.36	abnormal
E.S.	62	F	325	364	III	9.83	3.43	abnormal
M.Ö.	64	F	1210	500	III	3.46	1.56	abnormal
G.H.	53	M	1600	240	V	4.66	0.07	abnormal
H.B.	26	M	680	280	V	3.63	3.10	normal
I.W.	26	F	3800	195	V	3.80	0.04	normal
J.R.	32	M	1670	440	V	3.75	2.02	normal

Laboratory analyses were performed as described under Methods. Blood for H-TGL and LPL measurement was only drawn at plasma triglyceride concentrations below 5000 mg/100 ml.

at 10 U/kg body weight, in tubes containing a final EDTA concentration of 0.1%. Blood was immediately centrifuged at 4°C and plasma was removed. Only normal male donors were used except where otherwise stated. To obtain plasma for large scale purification of H-TGL, subjects were plasmapheresed 5 min after intravenous injection of heparin (50 U/kg of body weight) and blood was collected over a period of 15–25 min.

Subjects

Healthy male and female normal volunteers, all of whom had normal triglyceride and cholesterol concentrations, served as controls. Subjects ranged in age from 13 to 88 years, and were on a regular German diet. All subjects fasted 16 hr before blood samples were drawn for lipase assay. Laboratory and clinical data of patients with primary hyperlipoproteinemia are listed in **Tables 1** and **2.** Clinical data and laboratory findings of patients with familial LCAT deficiency have been previously reported (13). The lipoprotein pattern was determined as previously described (14). The descriptions Types I, III, and V were used according to the classification system of Fredrickson (15, 16). The diagnosis of Type III hyperlipoproteinemia was based on both typical clinical symptoms and the observation of lipoproteins of density $d < 1.006$ with β-lipoprotein mobility on electro-

TABLE 2. Clinical symptoms in patients with hypertriglyceridemia

Patient	Age	Sex	Episodic Abdominal Pain	Hepato-megaly	Spleno-megaly	Eruptive Xanthomata	Lipemia Retinalis	Pancre-atitis	Vascular Disease
U.K.	25	F	+	–	+	+	+	–	–
W.R.	23	M	+	–	+	+	–	+	–
W.C.	9	M	+	–	–	–	–	–	–
P.V.	9	M	+	–	+	+	+	–	–
J.B.	14	M	+	–	–	+	+	+	–
C.M.	17	F	+	–	–	+	–	+	–
C.A.	2	F	–	–	–	+	–	–	–
Mc.M.	9	F	+	–	–	–	–	+	–
A.K.	69	M	–	–	–	+[a]	–	–	+
E.S.	62	F	–	–	–	+[a]	–	–	+
M.Ö.	64	F	–	+	–	+[a]	–	–	+
G.H.	53	M	+	–	+	–	–	+	–
H.B.	26	M	+	+	–	–	+	+	–
I.W.	26	F	–	+	–	–	+	–	–
J.R.	32	M	+	+	–	–	–	+	–

[a] Tuberoeruptive xanthomas
All patients with Type I had first degree relatives with hypertriglyceridemia.

phoresis. All subjects with hyperlipoproteinemia were sampled as outpatients. None of the patients or normal subjects had received any medication known to affect lipid metabolism for at least two weeks before sampling.

Separation and partial purification of H-TGL and LPL

For experiments requiring the separated enzymes, the following procedure was employed. In this paper, buffer will refer to 0.005 M barbital-HCl pH 7.4, with 0.02% Na azide. All steps were carried out at 4°C. Separated and partially purified H-TGL and LPL were prepared as follows. Ten ml of postheparin plasma were diluted 1:2 with buffer and applied to a 2.2 × 5.5 cm column of Sepharose containing covalently linked heparin (Wilson Laboratories, Chicago, Ill.) (17) equilibrated with buffer. The column was then washed with 500 ml of buffer containing 0.2 M NaCl. This fraction contained no enzymatic activity. A stepwise elution was carried out using 300 ml of 0.75 M NaCl buffer and 300 ml of 1.5 M NaCl buffer. H-TGL elutes at approximately 0.7 M NaCl and LPL at approximately 1.5 M NaCl. The fractions with major enzyme activity (approx. 20 ml) were collected and dialyzed against buffer containing 0.2 M NaCl.

H-TGL protein for immunization was isolated as follows. Postheparin plasma (ca 300 ml) was precipitated by slowly adding with stirring an equal volume of $(NH_4)_2SO_4$ solution (60% saturated at 4°C). The solution was then centrifuged at 2,500 rpm for 20 min and the supernatant was dialyzed for 2 hr against two successive 10 liter volumes of buffer. The dialyzate was then diluted 1:2 with buffer and applied to a 5 × 25 cm heparin–Sepharose column equilibrated with buffer containing 0.2 M NaCl. The column was washed with 4 l of buffer containing 0.4 M NaCl. Both enzymes were eluted with buffer containing 1.5 M NaCl. The active fractions were pooled (ca. 700 ml) and diluted 1:1.5 with buffer and applied to a 2.5 × 9 cm concanavalin A–Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with buffer containing 1.0 M NaCl (18). The column was washed with 500 ml of buffer containing 1.0 M NaCl and both enzymes were eluted with buffer containing 0.5 M α-methyl-D-glucopyranoside (Fluka Chem., Neu-Ulm, Germany) and 1.0 M NaCl. The eluate (ca 700 ml) was dialyzed for 2 hr against buffer and applied to a 2.2 × 5.5 cm heparin–Sepharose column equilibrated with buffer containing 0.2 M NaCl. H-TGL was eluted with 500 ml of buffer containing 0.75 M NaCl and LPL with 500 ml of buffer containing 1.5 M NaCl. The active fractions were collected (ca 120 ml for each enzyme) and dialyzed extensively against 0.005 M $(NH_4)_2CO_3$, lyophilized, and stored at −20°C.

Triglyceride lipase assay

H-TGL and LPL isolated by heparin–Sepharose affinity chromatography were measured as described previously (8) except that 50 μl of preheparin plasma were used instead of 20 μg of apolipoprotein C II as cofactor for LPL.

Because previous experiments (9) with purified triglyceride lipase preparations had shown that H-TGL was maximally active at 1.0 M NaCl without cofactor and LPL at 0.1 M NaCl with either apolipoprotein C II or preheparin plasma as cofactor, two sets of conditions were chosen to provide maximal activity for H-TGL and LPL. Both assay systems contained in a total volume of 0.5 ml: 9.04 nmoles of glycerol-tri-[1-^{14}C]oleate (Amersham Buchler, Braunschweig, Germany), 55.3 miC/mmole); 8.5 μmoles of unlabeled triolein (Nu-Chek-Prep. Inc., Elysian, Minn., USA); 2.5 mg of gum arabic; 7.5 mg of defatted bovine albumin (BSA) (Behring Werke, Marburg, Germany); a final buffer concentration of 0.2 M Tris-HCl pH 8.4. The assay optimized for H-TGL contained 1.0 M NaCl. The assay optimized for LPL contained 0.1 M NaCl and 100 μl of preheparin plasma.

Selective measurement of H-TGL and LPL in postheparin plasma was carried out as follows: 120 μl of postheparin plasma were added to 40 μl of anti-H-TGL γ-globulin solution (15 mg/ml) and to 40 μl of 0.9% NaCl, respectively, and allowed to stand at 4°C for 30 min. LPL values were obtained by assaying 40 μl of the enzyme–anti-H-TGL suspension under LPL optimized conditions (0.1 M NaCl). H-TGL values were obtained by assaying 40 μl of the enzyme–0.9% NaCl suspension under H-TGL optimized conditions (1 M NaCl) and then subtracting the value obtained by assaying 40 μl of the enzyme–anti-H-TGL suspension under H-TGL optimized conditions (1.0 M NaCl). It was necessary to subtract this latter value because LPL has some activity (0–20%) when assayed at 1 M NaCl.

The substrate solution was prepared by sonification with a Branson sonifier-cell disruptor (Branson Instruments Co., Stamford, Conn., USA) of a solution of gum arabic, BSA, triolein and Tris buffer in a total volume of 100 μl/assay three times at 1 min with a setting of 3 (80 W) with cooling in ice. Care was taken not to foam the substrate solution. The assays were carried out in duplicate at 27°C, terminated as described previously (9) and FFA was determined

TABLE 3. Purification of hepatic triglyceride lipase for enzyme antibody production

Steps	Volume	Total Protein	Total TGL Activity	TGL Specific Activity	Purification	Recovery[a]	Overall Recovery
	ml	*mg*	*μmol FFA/h*	*μmol FFA/mg protein*	*fold*	%	%
Serum	300	17910	803	0.045	1	100	100
Supernatant of ammonium sulfate precipitation	560	11312	636	0.056	1.3	79.2	79.2
Heparin affinity column I	650	24.8	315	12.7	283	49.6	39.2
Concanavalin A chromatography	650	9.8	201	20.6	459	63.8	25.0
Heparin affinity column II	130	0.6	69.2	115	2875	34.4	8.6

[a] Recovery or loss for a given chromatographic step.

by the ion exchange resin method of Kelley (19). Enzyme activity was calculated as μmoles FFA/ml/hr. FFA release was linear with time for 1 hr or until 10–12% of the triglyceride was hydrolyzed.

Production and isolation of anti-H-TGL and anti-human γ-globulins

Rabbits were intramuscularly injected every two weeks with 0.5–1.0 mg of partially purified H-TGL protein or 0.2 ml of whole human plasma. The first injection contained 0.5 ml of Freunds complete adjuvant. The titer was checked 7 days after each injection by precipitation of H-TGL enzyme. When anti–H-TGL was detected, 30–50 ml of blood were collected every other week by severing a peripheral ear vein after irrigation with benzene. The γ-globulin fraction was isolated by Na_2SO_4 precipitation (20), dissolved in 0.9% NaCl solution, and stored at −20°C.

To determine cross reaction of anti-H-TGL with LPL, 1.0 ml of either H-TGL or LPL enzyme solution was mixed with an appropriate volume of either anti-H-TGL or anti-human γ-globulins (50 mg/ml) and the final volume brought to 1.25 ml with buffer containing 0.2 M NaCl. The solutions were allowed to stand 30 min at 4°C and then assayed.

Other methods

Protein was estimated by the method of Lowry et al. (21), with BSA as standard. Disc gel electrophoresis was performed in 7.5% polyacrylamide gel in the presence of sodium dodecyl sulfate by the method of Weber and Osborn (22). Bovine serum albumin was defatted as described by Chen (23). Cholesterol (24) and triglycerides (25) were determined as previously described. Plasma glucose values were obtained for all patients in the fasting state and 2 hr after oral administration of 100 g of glucose. Pathological tolerance tests were defined according to the Fajans–Conn criteria (2 hr, 140 mg/dl plasma or more).

Statistical Methods

Nonparametric tolerance limits (90% estimated with 95% confidence) were calculated from the tables of Somerville (26). A rank–sum test was used for comparison between different groups of normals and patients with lipid disorders (27).

RESULTS

Experimental data on the enzyme purification procedure are summarized in **Table 3.** A representative polyacrylamide gel electrophoretic pattern that shows the degree of purity of partially purified H-TGL used for enzyme antibody production is shown in **Fig. 1.**

Intramuscular injection of partially purified H-TGL protein into rabbits resulted in the production of an antibody against this enzyme. Complete precipitation of H-TGL could be achieved with antibody concentrations at which isolated plasma lipoprotein lipase activity was unaffected (**Fig. 2a** and **b**).

Measurement of lipase activity in whole postheparin plasma under H-TGL optimized conditions, i.e. at 1.0 M NaCl without cofactor, contained at least some LPL activity. This contribution by lipoprotein lipase was determined by precipitating H-TGL with the antibody and then measuring the residual activity at 1.0 M NaCl. This residual activity was subtracted from the lipase activity obtained at 1.0 M NaCl without antibody, yielding the value of H-TGL (**Fig. 3**). Release of both lipases was studied as a function of time at a dose of 10 U heparin/kg body weight (**Fig. 4**). Both enzymes reached a maximum at 10 min after heparin administration. As shown in Fig. 4, release and decay up to 60 min of both enzyme activites after heparin injection with 10 U/kg body weight were very similar. The effect of endogenous lipoproteins on postheparin plasma lipases was studied by mixing postheparin plasma with purified lipoprotein fractions and with pre-

heparin plasma obtained from patients with Type I and Type V hyperlipoproteinemia. Final VLDL or chylomicron triglyceride concentrations were 0.85, 1.70, 17.0, 21.2, 42.4, and 84.8 mM triglyceride/liter of plasma. It was found that concentrations of exogenous or endogenous triglyceride up to 60 mM in the plasma did not interfere with the assay.

The results obtained for H-TGL and LPL in the group of normals are summarized in **Fig. 5**. No attempt was made to correlate these data with age. Mean value for H-TGL in men was 5.42 ± 2.45 μmole FFA/ml/hr (SD) and 3.17 ± 1.49 μmole FFA/ml/hr (SD) in women, respectively. This sex difference was statistically significant ($P < 0.001$). H-TGL was measured in 15 patients with primary hyperglyceridemia and in 6 patients with familial lecithin-cholesterol-acyltransferase deficiency (Fig. 5a and b). The 8 patients with familial hyperchylomicronemia have not previously been reported. In all patients with Type I hyperlipoproteinemia values for H-TGL were within the 90% normal tolerance limit, and in all patients with Type V hyperlipoproteinemia or familial LCAT deficiency H-TGL values were within the 90% normal tolerance limit. All patients with Type III hyperlipoproteinemia had H-TGL values within the 90% normal tolerance limit.

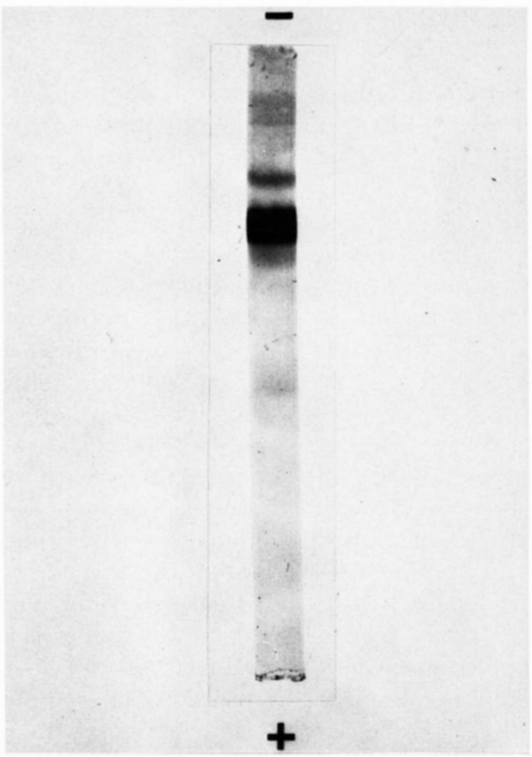

Fig. 1. Polyacrylamide gel electrophoresis of partially purified hepatic triglyceride lipase. The protein (80 μg/gel) was subjected to electrophoresis in buffer containing 1% sodium dodecyl sulfate and stained with Coomassie blue. Origin at top (cathode).

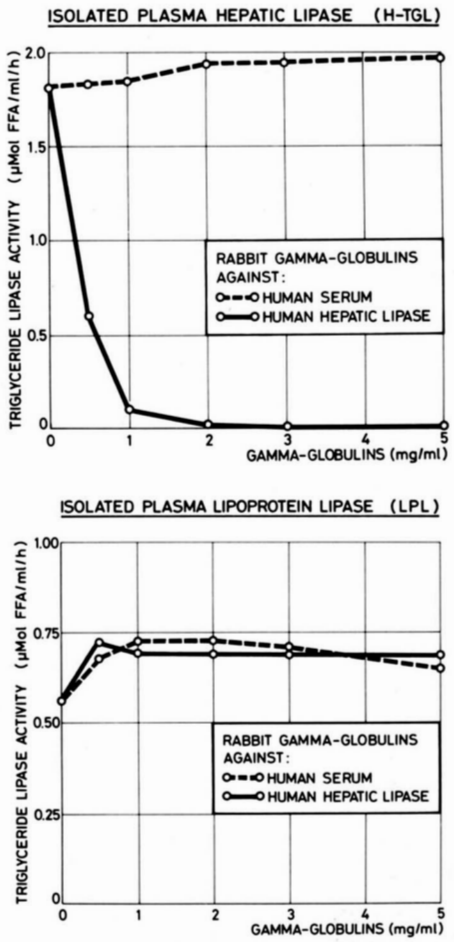

Fig. 2. (*a*) H-TGL and (*b*) LPL were separated and partially purified from postheparin plasma and incubated with increasing amounts of rabbit γ-globulins against H-TGL. The γ-globulin fraction from the serum of a rabbit immunized with preheparin serum from a normal human volunteer served as control. The concentration of γ-globulins is expressed in mg protein per ml incubation mixture. H-TGL was measured at 1.0 M NaCl and LPL at 0.1 M NaCl.

Mean value for LPL in men was 2.09 ± 1.18 μmole FFA/ml/hr (SD) and in women 1.76 ± 0.94 μmole FFA/ml/hr (SD). In the 12 patients with hyperchylomicronemia determination of plasma lipoprotein lipase by the enzyme antibody precipitation technique appeared to identify two populations of patients. Mean value of LPL in the patients with Type I was 0.25 ± 0.22 μmole FFA/ml/hr (SD). Seven out of eight patients with Type I had LPL values below the 90% normal tolerance limit ($P < 0.00001$) according to the White test (26). LPL measurement in 2 of the 4 patients with Type V was far below the 90% normal tolerance limit, while the remaining 2 patients had normal LPL activities. All patients with familial LCAT deficiency, as well as 2 of the 3 patients with Type

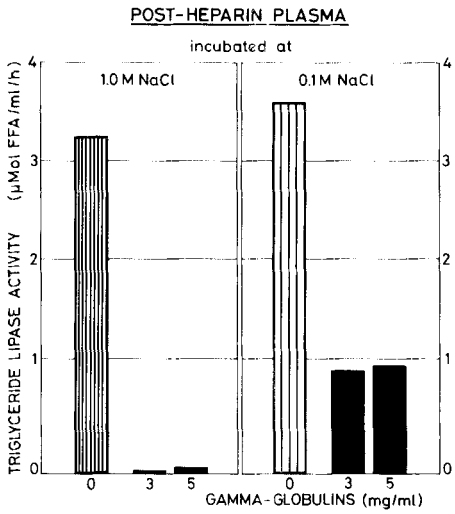

Fig. 3. Whole postheparin plasma was incubated with anti-H-TGL γ-globulins (black bars) and with 0.9% NaCl (striped bar) and assayed under H-TGL (1.0 M NaCl) and LPL (0.1 M NaCl) optimized conditions.

III hyperlipoproteinemia, had LPL levels within the 90% normal tolerance limit. The other Type III patient (A.K.) had a LPL value above the 90% normal tolerance limit.

DISCUSSION

Intravenous injection of heparin releases two lipases of hepatic and extrahepatic origin respectively (1–7). Separation and purification of these two plasma enzymes by affinity chromatography on Sepharose

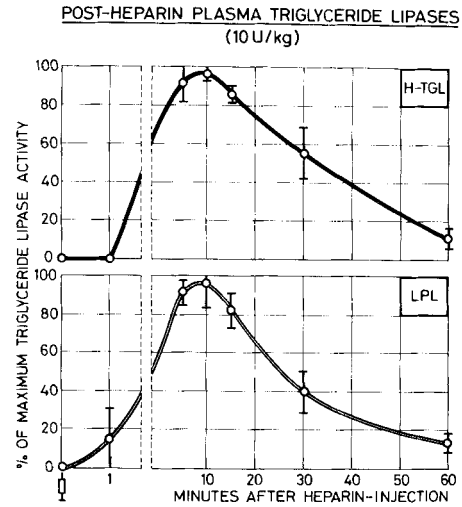

Fig. 4. The time course of H-TGL and LPL activities in human serum after intravenous injection of 10 U heparin per kg body weight. The curves represent mean values of four experiments carried out with one healthy normal subject on four consecutive days.

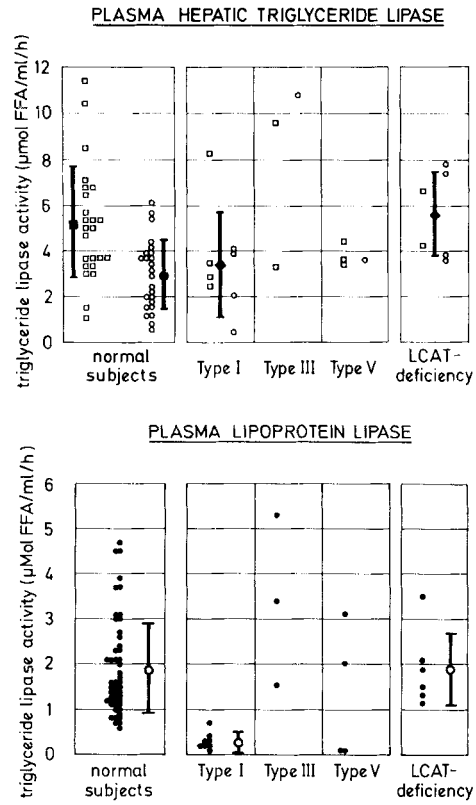

Fig. 5. The distribution of (*a*) H-TGL and (*b*) LPL activities in the studied population of normals and patients with lipid metabolism disorders. Males (□), females (○), males and females (●).

with covalently linked heparin and concanavalin A has enabled the characterization of the isolated enzymes (18). It could be shown that lipoprotein lipase requires apolipoprotein C II as cofactor for full activity. This enzyme is inhibited by both high sodium chloride and protamine sulfate concentrations (1, 6). H-TGL differs from LPL in all these characteristics. It is not dependent on an apolipoprotein cofactor for full activity (4), it requires high sodium chloride concentrations, and it is not inhibited by protamine sulfate (9). Both enzymes have similar molecular weights of approximately 65,000 and have been shown to be glycoproteins (18, 28). Though their exact role in lipoprotein metabolism has not been elucidated yet, selective measurement of the two enzymes appears to be important in determining the possible pathogenesis of lipid disorders. The use of an antibody which reacts with only one of these lipases allows specific measurement of H-TGL and LPL in whole postheparin plasma. This technique was applied in studying hypertriglyceridemia in patients with different lipoprotein disorders. The patients selected for this study either had severe hyperchylomicronemia alone or in combination with elevated pre-β-lipoproteins in the fasting state. Three patients had an

abnormal lipoprotein rich in triglyceride that is found in Type III hyperlipoproteinemia. Five of the six patients with documented familial lecithin-cholesterol-acyltransferase deficiency carried a low density lipoprotein rich in free cholesterol, lecithin, and triglycerides commonly designated as large molecular weight LDL (12). These particles most likely originate from chylomicrons and accumulate when lecithin-cholesterol-acyltransferase is missing.

The usefulness of a selective assay for hepatic and extrahepatic lipase activities in postheparin plasma has previously been demonstrated by Krauss, Levy, and Fredrickson (6). Their method was based on inactivation of lipoprotein lipase by protamine sulfate. The present method of investigating H-TGL and LPL separately in various forms of metabolic disorders with impaired triglyceride metabolism was based on precipitation of H-TGL by means of an antibody. The validity of such an assay was established by measuring lipase activity after incubation of the isolated and partially purified enzymes with rabbit γ-globulins against human hepatic lipase.

When this technique was applied to the study of hyperglyceridemia, it was found that all eight patients with a Type I lipoprotein pattern and two of the four patients with a Type V lipoprotein pattern had extremely low LPL levels with normal values for H-TGL. This is in agreement with observations by Krauss et al. (6). The fact that two patients who had elevations of both chylomicrons and pre-β-lipoproteins had low levels of lipoprotein lipase, as in the eight patients with hyperchylomicronemia alone, suggests a common pathogenetic mechanism for these two groups of patients and implies heterogeneity among patients with a Type V lipoprotein pattern. As suggested earlier (6), measurement of reduced LPL in patients with hypertriglyceridemia may serve as a useful laboratory test in distinguishing patients with such a biochemical defect from others in the same group who present a similar lipoprotein pattern.

Normal heparin-released lipases were found in the patients with floating β-lipoproteinemia as they were in the patients with familial LCAT deficiency. The accumulation of triglyceride-rich low density lipoproteins in the latter group of patients, therefore, cannot be explained by decreased levels of either H-TGL or LPL in post-heparin plasma.

In addition to quantitative measurement of these two enzyme activities, it will be necessary to investigate further the molecular properties of both enzymes as well as the exact mode of their interaction with circulating lipoproteins. These studies are presently under investigation in our laboratory.

We would like to thank Miss Brigitte Walter for her valuable technical assistance.

These studies were supported by a grant of the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 90, Cardiovasculäres System).

Manuscript received 18 July 1975; accepted 12 December 1975.

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