Effect of an anti-lipoprotein lipase serum on plasma triglyceride removal

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Abstract Anti-lipoprotein lipase sera injected intravenously in roosters blocked quantitatively the catabolism of very low density lipoprotein (VLDL) triglyceride. Antibodies were produced in rabbits immunized with highly purified lipoprotein lipase (LPL, glycerol ester hydrolase, E C 3.1.1.3) prepared from chicken adipose tissue. Following anti-LPL serum injection there was a linear increase in plasma triglyceride concentration. The rate of entry of triglyceride in plasma was estimated from the rate of triglyceride accumulation in the plasma of animals injected with anti-LPL serum, or from the disappearance curve of biologically labeled VLDL. In instances where both measurements were conducted in the same animals there was very close agreement between the two procedures. Inhibition of VLDL triglyceride catabolism of anti-LPL serum provided a way to characterize newly secreted VLDL that exhibited a broad spectrum of particle sizes with a median of 625 Å. They contained 76.2 ± 1.2% triglyceride and had a high ratio of free to ester cholesterol (2.46 ± 0.45). In control VLDL samples there was 46.1% triglyceride, and the ratio of free to ester cholesterol was 1.19. The complete inhibition of triglyceride removal by an antiserum prepared against adipose tissue LPL demonstrates that the NaCl-inhibited, serum-activated lipase prepared by affinity chromatography on heparin–Sepharose and concanavalin A–Sepharose columns is the enzyme responsible in vivo for the catabolism of VLDL triglyceride. Further, the kinetics of triglyceride accumulation in the plasma provide evidence that the site of degradation of VLDL triglyceride is within the plasma compartment.

Supplementary key words Lipoprotein lipase antiserum · very low density lipoprotein

There is considerable evidence that hydrolysis of triglycerides in chylomicrons and very low density lipoproteins is a necessary step in the transfer of triglyceride fatty acids to extrahepatic tissues (1). Circumstantial evidence indicates that the lipase present in extrahepatic tissues and referred to as lipoprotein lipase (glycerol ester hydrolase, EC 3.1.1.3) is the lipase responsible for this hydrolysis (1, 2). The functional site of action of the enzyme is presumed to be the surface of the endothelial cells of capillaries (1, 3). The aim of the present investigation was to verify that specific inhibition of lipoprotein lipase in the plasma compartment with an anti-adipose tissue lipoprotein lipase serum would indeed block triglyceride removal. Quantitative inhibition of plasma triglyceride degradation by a specific antibody prepared against adipose tissue LPL would provide evidence that lipoprotein lipases in various tissues share a common determinant. Further, an immediate blocking of triglyceride removal after intravenous injection of the antibody would be consistent with the current hypothesis that metabolism of very low density lipoproteins and chylomicrons occurs in the plasma compartment.

MATERIALS AND METHODS

Preparation of adipose tissue anti-lipoprotein lipase serum

Lipoprotein lipase was purified from rooster adipose tissue by a method similar to that described for the purification of porcine LPL (4). The major purification steps consisted of affinity chromatography on heparin–Sepharose and concanavalin A–Sepharose columns. Following the latter step the traces of concanavalin A still remaining in the preparation were removed by passage of the enzyme solution through an immuno-adsorbent column containing rabbit anti-concanavalin A immunoglobulin coupled to Sepharose 4B (3.5 mg protein/ml packed gel). The specific activity of highly purified enzyme preparations was 7385 ± 446 μeq fatty acid released/hr/mg protein (mean ± SEM, five preparations). Disc gel electrophoresis of purified enzyme in the presence of sodium dodecyl sulfate exhibited a single major component corre-

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Abbreviations: LPL, lipoprotein lipase; VLDL, very low density lipoprotein. The nomenclature of apolipoproteins employed is based on their COOH-terminal amino acids. In the terminology suggested by Alaupovic (9), they are defined as follows: apoLp-Ser, apoC-I; apoLp-Glu, apoC-II; apoLp-Ala, apoC-III.

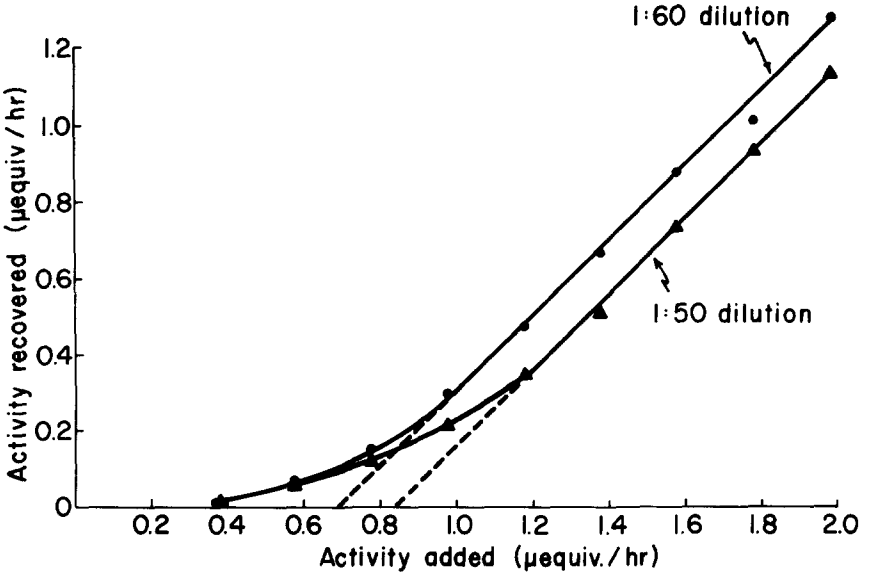

Fig. 1. Immunotitration of adipose tissue lipoprotein lipase by an anti-lipoprotein lipase serum. Increasing amounts of crude adipose LPL were incubated in a series of centrifuge tubes for 1 hr with 20 μl of anti-LPL serum diluted 1:50 or 1:60 with saline. The preincubation volume was 0.2 ml; preincubation volume contained 0.2 M NaCl, 30% glycerol, and 0.005 M Na veronal pH 7.0. After incubation, the tubes were centrifuged at 28,000 *g* for 30 min at 4°C. Enzymatic activity was measured in the supernatant fraction as indicated in the Materials and Method section. Parallel incubations were carried out with control rabbit serum.

sponding to a molecular weight of 60,000. The purified avian LPL exhibited the properties previously described for the porcine enzyme. It was stimulated sevenfold by the VLDL apolipoprotein ApoLp-Glu (maximal enzyme activity at 25 μg/ml of assay mixture); enzymatic activity was inhibited by ApoLp-Ser (50% inhibition, 18 μg/ml), ApoLp-Ala (50% inhibition, 80 μg/ml), ApoLp-Gln I (50% inhibition, 50 μg/ml), and ApoLp-Gln II (50% inhibition, 30 μg/ml). The enzyme was inhibited 90% by 1 M NaCl. Further details concerned with the purification and properties of avian lipoprotein lipases will be published elsewhere.[2]

The purified enzyme solutions were dialyzed against 40 volumes of distilled water at 4°C for 48 hr with four changes of the dialyzing solution. The enzyme preparations were concentrated by freeze-drying and solubilized in small volumes of 0.15 M NaCl before mixing with equal volumes of complete Freund's adjuvant. Immunization schedule consisted of biweekly to monthly injections of 100–300 μg of enzyme protein in the scapular and lumbar regions of rabbits over a period of 16 months. For the present studies sera from a single rabbit were utilized.

Titers were determined by immunotitration; increasing amounts of crude LPL extracted from avian adipose acetone powders with buffered salt solution (1.2 M NaCl, 0.005 M Na veronal, pH 6.5, 30% glycerol) were incubated in a series of centrifuge tubes for one hr with fixed quantities of diluted antiserum. In most instances the antiserum was diluted 1:40 with physiological saline and 20 μl volumes of diluted serum were titrated; the total incubation volume was 0.2 ml. Parallel incubations were carried out with control rabbit serum. In preliminary experiments it was established that optimal conditions for recovery of enzyme activity in the presence of control rabbit serum or for inhibition of activity by the antiserum were obtained when the incubation medium (0.2 ml) contained 0.2 M NaCl, 30% glycerol, and 0.005 M Na veronal, pH 7.0. After incubation, the tubes were centrifuged at 28,000 *g* for 30 min at 4°C. Enzymatic activity in the supernatant was determined as described previously (4) using [^{14}C]triolein emulsified with gum arabic as a substrate. Triolein containing [1-^{14}C]oleate in all three positions was purchased from DHOM Products, North Hollywood, California. The triolein specific activity was 1.78×10^5 dpm/μmole of triolein. Titers were determined graphically by plotting enzyme activity recovered as a function of enzyme added. The enzyme activity inhibited by 10 μl of diluted serum was measured by extrapolating the linear portion of the titration curve (**Fig. 1**). The three sera utilized in this

[2] Ming Whui Wang Yang and A. Bensadoun, unpublished data.

study had potencies equivalent to 250, 400, and 1634 μeq fatty acid/hr/ml of anti-serum.

Preparation of labeled lipoproteins

Biologically labeled very low density lipoproteins were prepared by injecting Na [1-^{14}C]oleate (100 μCi) bound to bovine crystalline albumin (5) into 18-hr fasted roosters. Thirty minutes later a large blood sample was collected by heart puncture. Clotting was prevented by EDTA (1 mg/ml of blood). VLDL was isolated from 40–60 ml of plasma by preparative ultracentrifugation at 40,000 rpm for 16 hr in a Beckman 60 Ti rotor. Before use in recipient animals, aliquots of the VLDL preparations were delipidated with chloroform–methanol (6) and the lipid classes were separated by thin-layer chromatography on silica gel H with a double-development procedure; this allowed a clear separation of triglycerides from free fatty acids (7). Counting of the radioactivity in the thin-layer strips (8) revealed that the triglyceride fraction contained 94.7–95.8% of the total VLDL radioactivity and the free fatty acid fraction 1.8–2.7%. Biologically labeled VLDL was utilized within 36 hr after its preparation.

Particle size determination

Very low density lipoproteins labeled in the triglyceride moiety with [1-^{14}C]oleic acid were classed by size using sucrose-gradient centrifugation (10). Centrifugations were performed in two 38 ml cellulose nitrate tubes (2.54 × 8.33 cm) in a SW 27 Beckman rotor at 20°C. Linear sucrose gradients (29.4–40%) were deposited into tubes with a Beckman gradient former. After delivery of the gradient (34.6 ml) the VLDL sample in a total volume of 1 ml of 50% sucrose was introduced at the bottom of the gradient with a syringe equipped with a long stainless steel needle. The round portion of the tube was filled with 2.4 ml of 60% sucrose. After centrifugation for 30–90 min at speeds between 10,000 and 25,000 rpm, 14 fractions corresponding to 0.5 cm thick layers were collected by suction from the sucrose–air interface. Aliquots of each fraction were counted in the presence of 10 ml of Aquasol counting solution (New England Nuclear Corp, Boston, Mass.). The migration of a particle in the gradient is given in the following expression of Stokes law:

$$\frac{dL}{dt} = \frac{d^2\omega^2 L(\rho_p - \rho_L)}{18\eta_L} \qquad \text{Eq. } 1$$

where d is the diameter of the particle, ω the speed of rotation, ρ_p the particle density, ρ_L and η_L the density and viscosity, respectively, of the sucrose solution at the distance L from the center of rotation. The particle density ρ_p was assumed to be 0.939 (10). The density and viscosity functions ρ_L and η_L were determined experimentally as described by Pinter and Zilversmit (10). With the conditions used, both ρ_L and η_L were linear functions of the distance L in the gradient.

$$\rho_L = 0.0053L + 1.0682 \quad \text{cgs units}$$

$$\eta_L = 0.0021L + 0.0091 \quad \text{cgs units}$$

These functions were substituted in Eq. *1*, which was integrated and solved for d. The equation obtained has the following form:

$$d = \sqrt{\frac{18}{t\omega^2}\, f(L)} \text{ cgs units} \qquad \text{Eq. } 2$$

In the above equation $f(L)$, a function of the distance L in the gradient, is determined by the characteristics of the sucrose gradient and is independent of t and ω.

$$f(L) = -0.0705\ \ln L - 0.3201\ \ln(0.1292 + 0.0053L) - 0.3077$$

Experimentally, the position of a sucrose layer in the gradient is measured by its height, h, above the densest level in the linear portion of the gradient. When using the protocol outlined above $L = 15.2$ cm $-h$ (cm).

The contributions of the acceleration and deceleration periods to $t\omega^2$ were determined graphically by plotting ω^2 against t and determining the area under the acceleration and deceleration curves.

Analytical procedures and determination of radioactivities

VLDL or plasma lipids were extracted and washed by the method of Folch, Lees, and Sloan Stanley (6) and separated by thin-layer chromatography on silica gel H using a solvent system of hexane–diethyl ether–acetic acid 90:10:1 or the double development method of Freeman and West (7). The first procedure was used for triglyceride specific activity measurement, the second for VLDL lipid composition. Lipid spots were visualized with iodine vapor. The lipid-containing areas were scraped off the plates, and the silica gel was transferred to glass columns and eluted with 75 ml of chloroform–methanol 2:1. Triglycerides, diglyceride, and monoglyceride were assayed by the method of Sardesai and Manning (11). Phospholipid phosphorus was determined by the method of Morrison (12). Total cholesterol was measured on lipid extracts, plasma, and lipoprotein fractions by the method of Zak et al. (13). Ratios of free to esterified cholesterol were determined by assaying fractions resolved by thin-layer chromatography. Cholesterol recovery

for the chromatography and elution steps was 92 ± 2% (mean ± SEM of 18 determinations).

Determination of triglyceride specific activity in VLDL solutions containing high concentrations of sucrose was conducted as follows. The fractions were dialyzed extensively against distilled water, freeze-dried, and then extracted with chloroform–methanol 2:1. Subsequent steps were similar to those described in the preceding paragraph. Extensive dialysis followed by thin-layer chromatography was necessary to avoid interference of sucrose with the triglyceride assay. Lipoprotein protein was determined by the method of Lowry et al. (14).

Animal experimental procedures

In preliminary experiments, the dose and schedule of antiserum injections necessary to block triglyceride removal were determined empirically by injecting levels of antiserum which would maintain a linear increase in plasma triglyceride.

In a first series of experiments, the effect of rabbit anti-LPL serum on triglyceride removal was studied in 4 five-month-old White Leghorn roosters (birds 1, 2, 3, and 4). The birds were fasted 18–20 hr before the experiment. Anti-LPL serum or control rabbit serum was injected in conscious birds via a polyethylene catheter in the wing vein as follows. At time 0 the birds received 2.5 ml of serum/kg body wt. This was followed by booster injections of 1 ml/kg body wt at 30, 60, and 90 min. Biologically-labeled VLDL was injected at t=30 min. Four-ml blood samples were withdrawn at 0, 30, 60, and 90 min for triglyceride specific activity measurements. Blood volume was maintained by injecting an equal volume of physiological saline. At 120 min, a large blood sample for VLDL characterization was withdrawn by heart puncture.

In a second set of experiments, removal of labeled VLDL was first examined in the absence of anti-LPL serum and then, in the same animal, after injection of anti-serum. The following protocol was followed in three roosters fasted for 18 hr (birds 5, 6, and 7). At time 0, biologically-labeled VLDL, prepared as outlined above, was injected through a polyethylene catheter in the wing vein. Three-ml blood samples were withdrawn at close time intervals for 30 min. At 30 min, 2.5 ml of anti-LPL serum/kg body wt was administered intravenously; this was followed by injections of 1 ml of anti-serum/kg body wt at 60 and 90 min. After anti-serum injection blood samples were withdrawn at 30 min intervals. At 120 min a large blood sample for VLDL characterization was withdrawn by heart puncture.

Biologically-labeled VLDL examined for particle size distribution was prepared in roosters 8 and 9. At time 0, 2.5 ml of anti-serum/kg body wt was injected intravenously through a polyethylene catheter inserted in the wing vein. Booster injections, 1 ml of anti-serum/kg body wt, were administered at 30 min and 60 min. [1-^{14}C]Oleic acid (20 μCi from DHOM products, North Hollywood, California) coupled to albumin (5) was injected intravenously either at t=0 (bird 8) or at t=30 (bird 9). Blood samples were withdrawn at 0, 60, 90, and 120 min. The last blood sample was obtained by heart puncture and was utilized for the preparation of VLDL samples for particle size determination and chemical analyses.

RESULTS

Effects of anti-adipose LPL serum on plasma triglyceride removal

In the first series of experiments (**Fig. 2**) administration of anti-LPL serum caused a dramatic linear rise in total plasma triglyceride. One hundred and fifty min after anti-LPL serum injection, plasma triglyceride concentrations increased 7- and 10-fold, respectively, in experimental birds 1 and 4 (Fig. 2). Plasma triglyceride concentrations of control birds 2 and 3 remained essentially constant during the experimental period. Biologically-labeled VLDL injected intravenously 30 min after the first anti-serum dosage disappeared at a very slow rate from the plasma compartment. Following removal of 12–24% of the initial dose during the first 60 min, VLDL radioactivity in the plasma remained essentially constant. In the control birds, labeled VLDL disappeared with a very short half-life as has been previously reported for other species (15).

In the second series of experiments the pattern of removal of labeled VLDL was examined in the same bird, first in the absence and then in the presence of anti-LPL serum. Three experiments were conducted (birds 5, 6, and 7). Results for bird 6 are presented in **Fig. 3.** Confirming the first series of experiments, plasma triglyceride concentration rose linearly after anti-LPL serum injection. Between 60 and 140 min triglyceride radioactivity in the plasma remained constant. Radioactivity present in the plasma was sufficient to be estimated with low error. The mean measured radioactivity was 862 ± 16 cpm. The SEM is of the same order of magnitude as the expected counting error.

Plasma triglyceride production rates

Table 1 presents a summary of triglyceride production rates measured in birds with different initial plasma triglyceride levels. Production rates were cal-

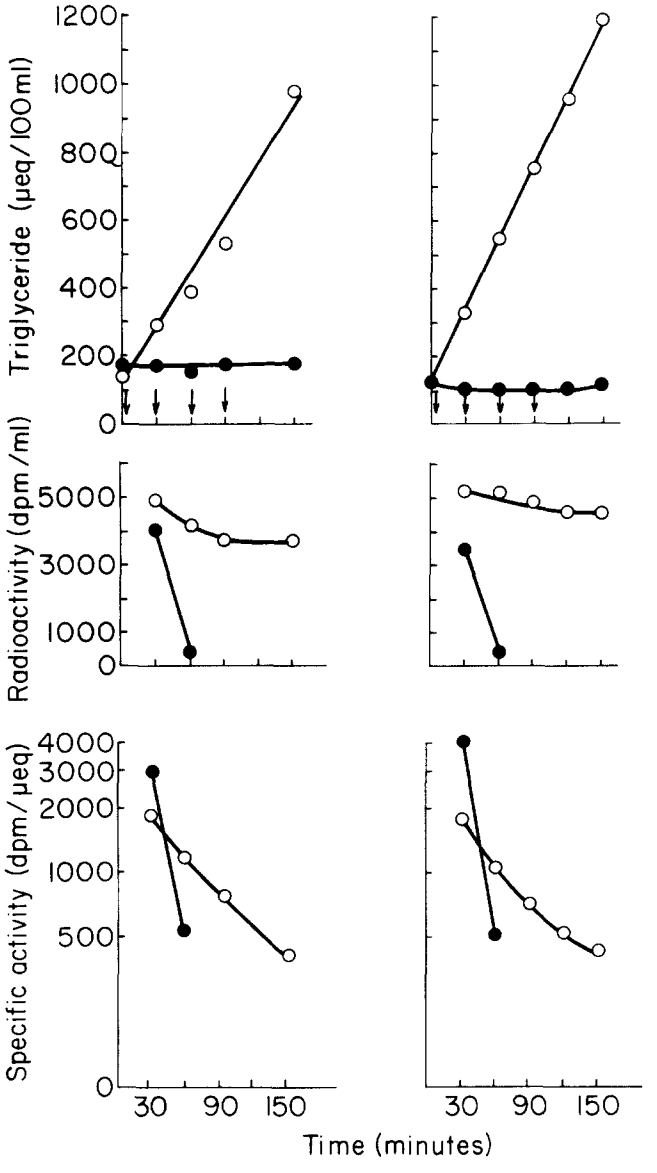

Fig. 2. Inhibition of plasma triglyceride removal by anti-LPL serum. Anti-LPL serum (○-○-○-○) or control rabbit serum (●-●-●-●) was injected in 18-hr fasted roosters. Antiserum had a titer equivalent to 250 μeq fatty acid/hr/ml of antiserum. Antiserum injections are indicated by arrows. On the left are data for experimental bird 4 and control bird 3; on the right, data for experimental bird 1 and control bird 2.

culated either from the rate of triglyceride accumulation in the plasma after anti-serum injection or from the rate of disappearance of a dose of biologically-labeled VLDL. Birds 1, 2, 3, and 4 had similar body weights and initial plasma triglyceride concentrations; their triglyceride production rates would be expected to be similar. Production rates measured by the antibody (birds 1, 4) or the isotopic (birds 2, 3) methods were in very close agreement. In the three instances (birds 5, 6 and 7) where the two methods were employed in the same animals, triglyceride entry rates into the plasma were essentially identical.

Characterization of newly secreted VLDL

Table 2 summarizes the composition of VLDL (d < 1.006) isolated from blood collected 2 hr after anti-

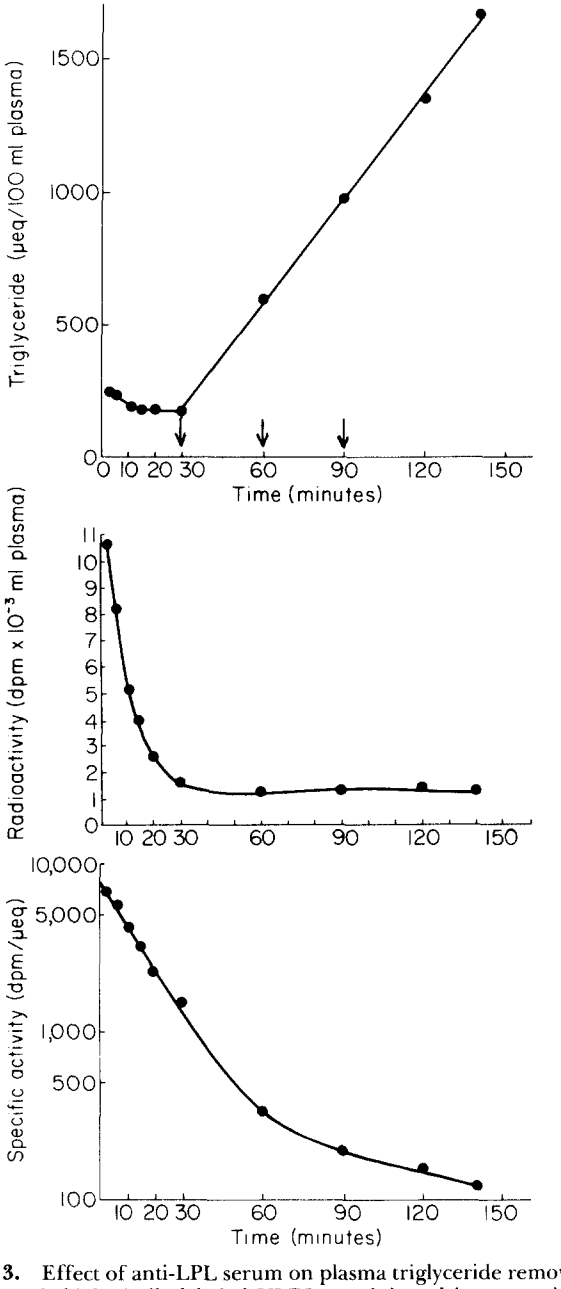

Fig. 3. Effect of anti-LPL serum on plasma triglyceride removal. At time 0, biologically labeled VLDL was injected in a conscious rooster (bird 6) via a wing vein catheter; blood samples were withdrawn at close time intervals for 30 min; at t=30 min, 2.5 ml of anti-LPL serum/kg body wt was administered intravenously; this was followed by injections of 1 ml of anti-serum/kg body wt at 60 and 90 min. Anti-LPL serum had a titer equivalent to 1634 μeq fatty acid released/hr/ml serum. Antiserum injections are indicated by arrows.

LPL serum injection. As would be expected the triglyceride content is strikingly higher than that of VLDL prepared from plasma of control fasted roosters. The relative amount of cholesterol is lower and the free to esterified cholesterol ratio higher than in control VLDL samples. The free esterified cholesterol ratio is probably underestimated in newly secreted particles, since partially degraded VLDL was present at the time of anti-LPL serum injection.

Particle size distribution of newly secreted VLDL was determined on two pooled VLDL samples (birds 8 and 9) by flotation in sucrose density gradients (**Fig. 4**). The VLDL particles were labeled in vivo by incorporation of [1-^{14}C]oleic acid into the triglycerides. Triglyceride radioactivity was employed as a marker for triglyceride mass. This method was validated by demonstrating that the bottom (less than 600 Å), middle (600–900 Å), and top (over 900 Å) portions of the sucrose gradients had similar triglyceride specific activities. For these three particle size intervals, the specific activities were 9264, 9097, and 9727 dpm/μmole of triglyceride. In a second VLDL sample studied the triglyceride specific activities for the top, middle, and bottom fractions were 5247, 6109 and 5270. The median sizes for the two VLDL samples studied were 600 and 650 Å. In both instances the newly secreted particles exhibited a broad spectrum of particle sizes. The 90th percentiles were respectively 975 and 1000 Å.

TABLE 1. Plasma triglyceride production rates determined by two procedures[1]

Animal	Body Weight	Plasma Triglyceride	Production Rates: Antibody Method	Production Rates: Isotopic Method
	g	*μeq/100 ml*	*μeq triglyceride/100 ml plasma/hr*	
1	2260	136	347	
2	2250	172		460
3	2490	123		421
4	2260	123	429	
5	2058	53	172	170
6	1097	240	1010	990
7	1095	47	107	105
8	3040	43	99	
9	2440	31	211	

[1] Plasma triglyceride production rates were calculated from the rate of triglyceride accumulation in the plasma following anti-LPL serum or from the rate of disappearance of intravenously injected labeled VLDL. In the latter case production rates were calculated as follows. The fractional turnover rates were calculated from the first order triglyceride specific activity functions determined during the first 30 min after injection of the labeled VLDL. Production rates were obtained by multiplying the fractional turnover rate by the mean plasma triglyceride concentration.

DISCUSSION

This study documents the ability of adipose tissue LPL to induce antibodies in rabbit. The inhibition of triglyceride removal in vivo by an antiserum prepared against adipose tissue LPL demonstrates that the salt-inhibited, serum-activated lipase prepared by affinity chromatography according to the protocol outlined in the method section is the enzyme responsible in vivo for the catabolism of VLDL triglyceride. Since there was a total inhibition of plasma triglyceride removal it can be concluded that quantitative transfer of VLDL triglyceride to tissues requires the presence of an enzymatically active LPL. Furthermore, the data would support the view that LPL in various tissues must be structurally similar. In immunological studies by the techniques of double diffusion LPL extracts from adipose, heart, and ovarian tissues exhibit lines of identity when reacted against rabbit anti-adipose LPL. Similarly, heart and adipose LPL enzymatic activities are completely inhibited in vitro by the same anti-serum.[2] Thus, both the in vivo and in vitro data suggest that LPL of various tissues share a common immunological determinant.

TABLE 2. Lipid and protein composition of newly secreted VLDL

	Protein	VLDL Composition: PL	MG	DG	TG	C	Ratio of Free to Ester Cholesterol
		%					
Newly secreted VLDL[a]	10.5 ± 1.2	8.3 ± 1.2	0.2 ± 0.1	0.3 ± 1.2	76.2 ± 1.2	4.8 ± 0.6	2.46 ± 0.45
Control VLDL[b]	12.9	27.3			46.1	13.6	1.19

[a] Figures represent means ± SEM of analyses of six samples of VLDL ($d < 1.006$) prepared from plasma obtained 2 hr after anti-LPL serum injection in six roosters.
[b] Average composition of two plasma VLDL samples isolated from blood of control roosters fasted 18 hr.
Abbreviations: PL, phospholipid; MG, monoglyceride; DG, diglyceride; TG, triglyceride; C, total cholesterol.

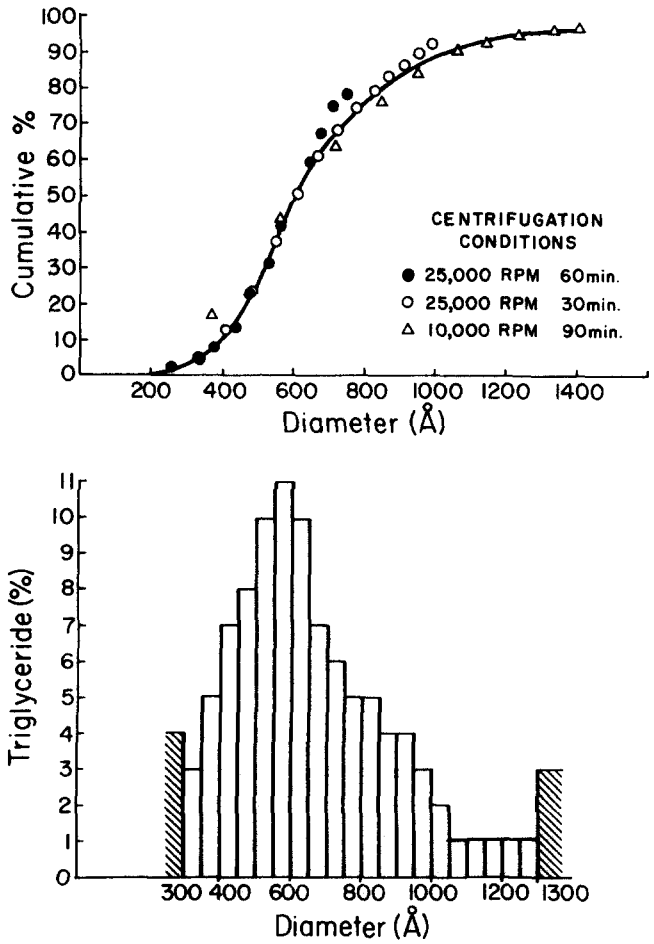

Fig. 4. Particle size distribution of newly secreted VLDL. A rooster (bird 8) was injected intravenously at t = 0 with [1-^{14}C]oleic acid (20 μCi) coupled to albumin. Anti-LPL serum (equivalent to 440 μeq fatty acid released/hr/ml serum) was administered at t=0, 60, 90, and 120 min. VLDL (d < 1.006) was prepared from blood obtained by heart puncture at t=120 min. Particle size distribution of [^{14}C]triglyceride-labeled VLDL was obtained by flotation in sucrose density gradients. Three centrifugation conditions were employed; the upper graph presents data on a cumulative basis for three centrifugation runs; the lower graph presents the same data as a histogram.

When labeled VLDL was injected into birds which had been pretreated with anti-serum (Fig. 1) 15–20% of the radioactive VLDL was removed during the first 60 min; during the next 60 min plasma triglyceride radioactivity remained constant. However, during these two 60-min intervals the rate of triglyceride accumulation was constant. The initial removal of labeled VLDL might represent removal of denatured VLDL (removal by the reticulo-endothelial system) or equilibration with an extravascular pool. In the pig, Sniderman, Carew and Steinberg (16) reported an extravascular LDL pool that was 20–30% of the size of the plasma LDL pool. The possibility that some labeled VLDL was bound on endothelial surfaces of tissue capillary beds as VLDL–LPL antibody complexes must also be considered.

The availability of anti-LPL serum provided a means of characterizing newly secreted VLDL collected in vivo in conscious animals. The term newly secreted VLDL is preferred over nascent VLDL since the latter term was used for lipoproteins isolated from the Golgi apparatus (17). Triglyceride-rich lipoproteins are thought to decrease in size in the course of their catabolism (18). Consonant with this view was the finding that the median particle size of newly secreted VLDL (diameter 600 Å) was 50% larger than that of normal VLDL samples (19). VLDL triglyceride was secreted in a broad spectrum of particle diameters. Similar observations have been reported for chylomicrons prepared from dog and rat lymph (20). In the present study 20% of the triglyceride in VLDL samples was present in particles with diameters smaller than 450 Å. Thus in control animals, particle size of a VLDL particle is not per se an index of its catabolic history. VLDL particles in a narrow size interval have a complex origin; some are newly secreted lipoproteins, others are lipoproteins that have lost varying proportions of their original triglyceride.

The present study provides information on the site of action of LPL. In the anti-LPL serum-treated animals initial triglyceride accumulation occurred at the time of anti-serum injection. This instantaneous blocking of triglyceride removal provides direct evidence that the functional LPL responsible for VLDL triglyceride hydrolysis is located in sites within the plasma compartment or in extravascular pools readily accessible to immunoglobulins.

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