Separation of molecular species of lipoprotein lipase from adipose tissue

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Abstract When NH_4OH–NH_4Cl extracts of adipose acetone powder were applied to agarose gel chromatography columns, two peaks of lipoprotein lipase were eluted. The first activity peak (LPL_a) was eluted with an elution volume of a protein of molecular weight approximately five times that of the second (LPL_b). Addition of heparin to the eluted fractions markedly stimulated activity of LPL_a, but suppressed that of LPL_b. Both lipases had the characteristics that distinguish lipoprotein lipase from other tissue lipases: a requirement for serum for substrate activation, inhibition by 1 M NaCl, and an alkaline pH optimum (pH 8.0). It is concluded that these fractions represent two species of lipoprotein lipase.

Supplementary key words gel filtration · heparin · serum activation

TRANSPORT of lipoprotein triglyceride from the circulation across cell membranes to the adipocyte depot requires prior hydrolysis of the triglyceride to glycerol and fatty acid (1, 2). In adipose tissue the hydrolysis is catalyzed by lipoprotein lipase. This enzymatic activity has consistently been shown to be directly proportional to triglyceride uptake by the tissue in a variety of nutritional and hormonal states (3–5).

There is experimental evidence suggesting that a portion of the adipose tissue lipoprotein lipase activity is located at the lumen surface of the capillary endothelium and that it is this activity that accounts for the uptake of triglyceride into the tissue (6, 7). However, the adipocyte has been implicated in synthesis (8) and secretion of the enzyme into the interstitial space (9). Presumably, this secreted enzyme is then further transported to the capillary surface. We have begun to explore the possibility that lipoprotein lipase located at various sites in the tissue may have different properties and functions. In this initial study, only lipoprotein lipase of intact adipose tissue was investigated. Our findings suggest that at least two forms of this enzyme exist which can be distinguished by molecular size and interaction with heparin.

MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing 250–350 g, were used in these experiments. In order to maximize enzyme activity, in this initial study we have used fed and refed rats, sometimes supplementing the chow diet with 10% glucose in the drinking water in order to further increase lipoprotein lipase activity.

The rats were decapitated and the epididymal adipose tissue was quickly excised. Acetone powders were prepared from approximately 7 g of adipose tissue by homogenizing the tissue in 100–200 ml of acetone in a Waring Blendor for 1 min at ambient temperature. The homogenate was filtered onto Whatman no. 5 filter paper and washed briefly with additional aliquots of acetone and ethyl ether. The air-dried acetone powders were either extracted immediately or stored at $-20°C$ with desiccant for several days before extraction.

Extracts of these acetone powders in 0.05 M NH_4OH–NH_4Cl buffer (pH 8.6) were the sources of enzyme activity. The extracts were prepared by homogenizing acetone powders in this buffer in a Ten Broeck glass tissue grinder in ice. All further procedures were carried out at 4°C. The initial extracts were centrifuged at 2000 g for 20 min to remove the insoluble material. The supernatant fraction was then concentrated through an Amicon UM-10 membrane to 2–3 ml. After concentration the extract was centrifuged at 12,000 g for 20 min to remove

any precipitates. This supernatant fraction was applied by gravity flow to a gel bed of Sephadex G-200 (Pharmacia) or Bio-Gel A-1.5M (8% agarose, Bio-Rad) equilibrated with 0.05 M NH$_4$OH–NH$_4$Cl buffer (pH 8.6), eluted with the same buffer, and assayed for LPL activity by a radioactive method previously described (10).

Substrate was prepared by sonicating the following reagents in a total volume of 9.0 ml: 1.35 mg of triolein containing approximately 2 μCi of glyceryl tri[1-^{14}C]-oleate, 3.0 ml of serum from a dog fasted overnight, 0.9 ml of 1% Triton X-100, 0.3 ml of 5% bovine serum albumin (pH 8.0), 0.8 ml of 2 M Tris–HCl buffer (pH 8.2), and 4.0 ml of H$_2$O. 0.6 ml of the sonicated substrate was incubated with 0.4-ml aliquots of enzyme for 60 min. The final pH of the assay mixture was 8.0. The results are expressed as counts per minute per aliquot. When the effect of heparin on activity was determined, 1.9 μg of heparin (Calbiochem, Los Angeles, Calif., 170 units/mg) was added.

The elution volume (V_e) was measured from the start of sample application to the half-height point of the rising edge of the effluent peak (11), and the void volume (V_o) was determined by measuring the V_e of a solute, tobacco mosaic virus (mol wt 40.7 $\times$ 10^6), incapable of penetrating the gel matrix. The chromatographic columns employed were 1.5 $\times$ 90 cm. Volumes of the gel bed, flow rates, void volumes, and fraction volumes are given in the legend to each figure. Proteins for calibration of the columns were purchased from Sigma Chemical Co. (bovine thyroglobulin, type 1, and ovalbumin grade V) and Mann Chemical Co. (γ globulin, fraction II). Tobacco mosaic virus was the gift of Dr. Samuel G. Wildman, UCLA. Protein was determined by the method of Lowry et al. (12), and the results are expressed as absorbance units at 500 nm.

RESULTS

Effect of heparin on lipoprotein lipase eluted from Sephadex G-200 columns

Initially, using columns of Sephadex G-200 gel, we examined the elution pattern of lipase activity from extracts of intact adipose tissue. Enzyme activity was eluted as a single peak in the volume of excluded proteins with a trailing portion which sometimes appeared as a smaller second peak. Since only a portion of the lipoprotein lipase in intact adipose tissue is released by heparin, we suspected that the enzyme not released might react differently to this mucopolysaccharide. Therefore, the fractions eluted from the gel column were assayed for lipoprotein lipase activity in the presence and absence of heparin. A consistent finding was stimulation by heparin of the ascending portion

of the peak of activity and inhibition of the descending portion.

Having observed this stimulation and inhibition of lipase activity, we studied the effect of heparin concentration. The increase in lipoprotein lipase activity with the initial heparin concentration (1.9 μg/0.4 ml) was also observed with lower and higher concentrations. On the other hand, the decreased activity was apparent only at concentrations of 1.9 and 2.4 μg. At lower concentrations of heparin, stimulation of activity was observed.

Separation of two peaks of lipase activity on Bio-Gel A-1.5M

The experiments with Sephadex G-200 suggested that a gel with a higher porosity might separate the single peak of lipase activity into its components. Bio-Gel A-1.5M has a molecular weight exclusion limit for globular species of 1.5 $\times$ 10^6. When extracts of adipose tissue were placed on this agarose gel, two peaks of lipoprotein lipase activity were eluted: an initial large peak in the volume of excluded proteins was followed by a second peak usually with about half the activity of the first. Activity of the first peak eluted was stimulated by addition of heparin, whereas the activity of the second smaller peak was markedly suppressed (Fig. 1). Probably the activity previously eluted from the Sephadex gel represented the sum of these two activities. While recovery of protein in the gel filtration procedure was routinely virtually 100%, recovery of enzyme activity varied from
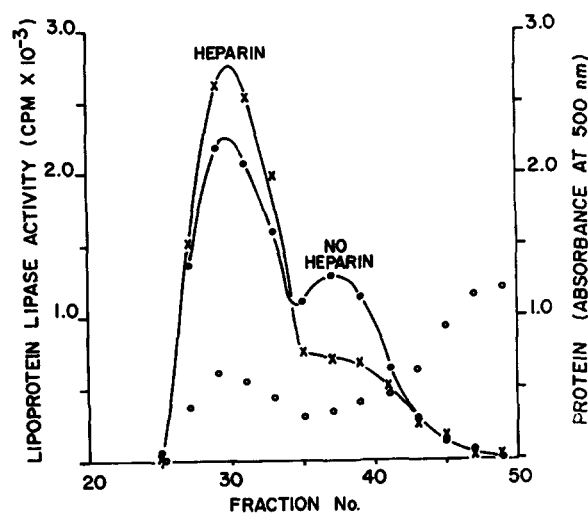

Fig. 1. Effect of heparin on lipoprotein lipase activity eluted from Bio-Gel A-1.5M. 60 mg of protein, extracted in 0.05 M NH$_4$OH–NH$_4$Cl buffer (pH 8.6) from adipose acetone powder, was applied to a Bio-Gel A-1.5M column (1.5 $\times$ 80 cm) equilibrated with the same buffer. 1.5-ml fractions were collected at the rate of 4.5 ml/hr, the void volume (45 ml) corresponding to fractions 1–27. 0.4-ml aliquots were assayed for lipoprotein lipase activity with (X) and without ($\bullet$) 1.9 μg of heparin. Results are expressed as cpm/0.4 ml aliquot. Protein (O) was determined colorimetrically on 0.1-ml aliquots and is expressed as absorbance units/0.1 ml.

approximately 50 to 100%. Recovery was calculated from the total enzyme activity and protein applied to the column and the total amounts in the eluted fractions.

Characterization of the two lipolytic activities as lipoprotein lipases

Criteria usually employed to identify lipoprotein lipase in crude tissue extracts are the requirement for a lipoprotein substrate, inhibition by 1 M NaCl, and an alkaline pH optimum. To determine if both lipolytic activities separated on agarose columns did indeed have the characteristics of lipoprotein lipase, the criteria above were examined. The pH maxima for the two activities appear to be the same, approximately pH 8.0 (Fig. 2). Both activities were markedly inhibited by the removal of serum from the assay system (Fig. 3) and by the addition of even small amounts of NaCl (Fig. 4). The smaller lipase moiety showed a somewhat greater sensitivity to NaCl, but both were essentially completely inhibited by 1 M NaCl (Fig. 5). Since both lipases have thus been characterized as lipoprotein lipases, we have adopted the subscripts "a" and "b" to designate the two species, LPL_a referring to the larger entity and LPL_b to the smaller one.
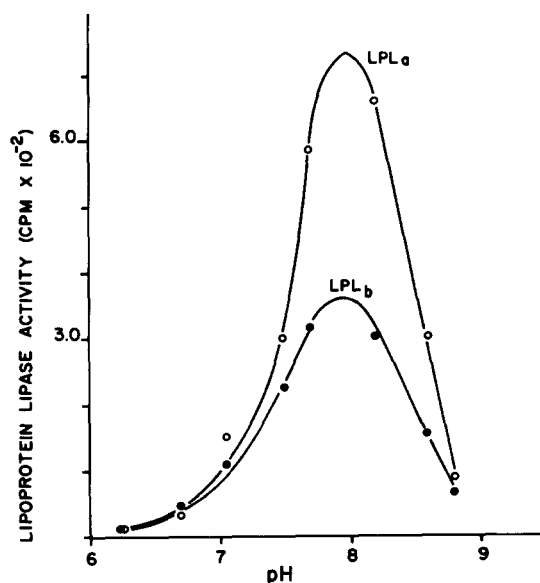

FIG. 3. Characterization of LPL_a and LPL_b: effect of serum on activities. 26 mg of protein, extracted in 0.05 M $NH_4OH–NH_4Cl$ buffer (pH 8.6) from adipose acetone powder, was applied to a Bio-Gel A-1.5M column (1.5 × 80 cm) equilibrated with the same buffer. 1.5-ml fractions were collected at the rate of 4.5 ml/hr, the void volume (45 ml) corresponding to fractions 1–18. 0.4-ml aliquots of the collected fractions were assayed with the standard substrate (X) and with a substrate in which the serum component was replaced by 0.15 M NaCl (●). The results are expressed as cpm/0.4 ml.

FIG. 2. Characterization of LPL_a and LPL_b: pH optima. 67 mg of protein, extracted in 0.05 M $NH_4OH–NH_4Cl$ buffer (pH 8.6) from adipose acetone powder, was applied to a Bio-Gel A-1.5M column (1.5 × 80 cm) equilibrated with the same buffer. Lipoprotein lipase activity of the eluted fractions was determined, then fractions from the ascending edge of the first activity peak were combined (LPL_a), as were those from the descending edge of the second activity peak (LPL_b). 0.4-ml aliquots were assayed for lipoprotein lipase activity. The pH of the assay mixture was adjusted by varying the HCl content of the Tris–HCl buffer component. pH values shown were measured after the addition of 0.4-ml enzyme aliquots to the assay mixture. Results are expressed as cpm/0.4 ml.
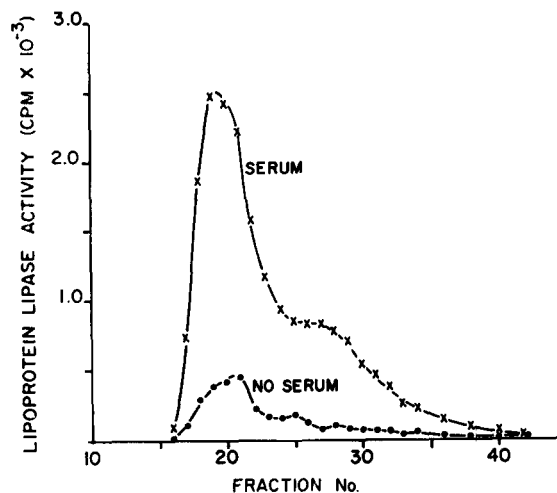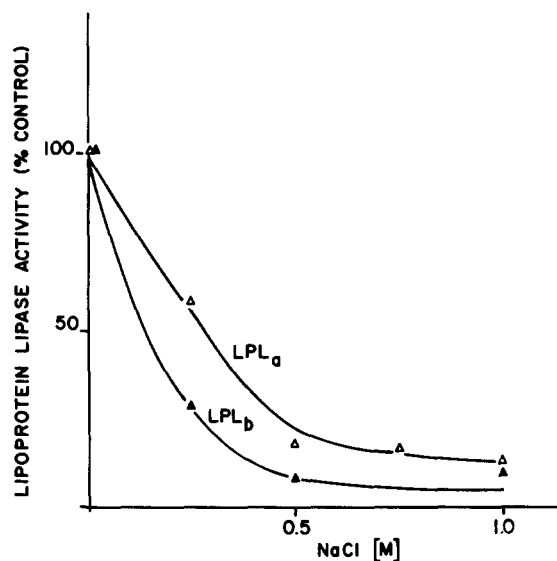

FIG. 4. Effect of NaCl concentration on LPL_a and LPL_b. From a separation of LPL_a and LPL_b by gel filtration (Fig. 5) representative samples of the two activities were selected, fractions 18, LPL_a, and 28, LPL_b. 0.3-ml aliquots were incubated at 4°C with 0.1 ml of different concentrations of NaCl. After 30 min, substrate was added and lipoprotein lipase activity measured. Results are expressed as % of the activity with no addition of NaCl.

Further characterization of LPL_a and LPL_b

Stability to incubation at 37°C. Since the lipoprotein lipase activity of adipose tissue extracts has been known to decrease on incubation at 37°C (13), it was of interest to determine whether there was a difference in stability
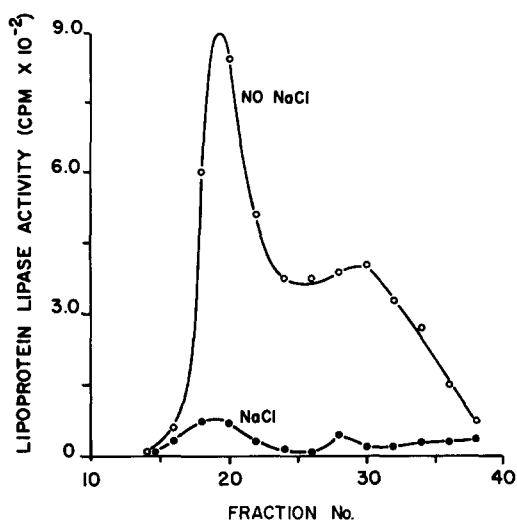
FIG. 5. Characterization of LPL$_a$ and LPL$_b$: effect of 1 M NaCl on activities. 74 mg of protein, extracted in 0.05 M NH$_4$OH–NH$_4$Cl buffer (pH 8.6) from adipose acetone powder, was applied to a Bio-Gel A-1.5M column (1.5 × 80 cm). 1.8-ml fractions were collected at the rate of 5.4 ml/hr, the void volume (45 ml) corresponding to fractions 1–17. 0.3-ml aliquots were incubated at 4°C with either 0.1 ml of NH$_4$OH–NH$_4$Cl buffer (○) or 0.1 ml of 4 M NaCl in NH$_4$OH–NH$_4$Cl buffer (●). After 30 min, substrate was added and the lipoprotein lipase activity measured. Results are expressed as cpm/0.4 ml.

of LPL$_a$ and LPL$_b$ at that temperature. When aliquots of LPL$_a$ and LPL$_b$ were incubated at 37°C for 30 and 60 min, the rates of inactivation of both species were essentially the same (Table 1). Further, as shown also in Table 1, the degree of inhibition or activation of the residual activities by heparin remained constant after partial heat inactivation. Thus, LPL$_a$ and LPL$_b$ in this partially separated state could not be distinguished by this means.

TABLE 1. Stability of LPL$_a$ and LPL$_b$ activities to incubation at 37°

Enzyme	Heparin (1.9 μg)	Lipoprotein Lipase Activity		
		Time (min)		
		0	30	60
		cpm × 10⁻²		
LPL$_a$	−	4.0	3.4	3.1
	+	5.8	4.5	4.1
Ratio +/−		1.4	1.3	1.3
LPL$_b$	−	1.4	1.1	0.92
	+	0.78	0.57	0.48
Ratio +/−		0.54	0.51	0.52

An NH$_4$OH–NH$_4$Cl extract of adipose acetone powder was applied to a column of Bio-Gel A-1.5M (1.5 × 80 cm). Fractions from the ascending edge of the first activity peak were combined (LPL$_a$) and also those from the descending edge of the second activity peak (LPL$_b$). 0.4-ml aliquots were incubated at 37°C for the times shown. Then substrate was added and lipoprotein lipase activity determined with and without 1.9 μg of heparin per aliquot. Results are expressed as cpm/0.4 ml and are representative of one of two similar experiments. The activities are the means of duplicate determinations.

Interrelationship of LPL$_a$ and LPL$_b$. To determine whether the two species of lipoprotein lipase were interconvertible under the conditions of our experiments, we collected the activity peaks from an initial separation, concentrated the samples and rechromatographed each peak separately. LPL$_a$ was recovered as a single heparin-stimulated peak (Fig. 6). In contrast, when LPL$_b$ was recycled, two peaks of activity were eluted from the column (Fig. 7). One of the activities from recycled LPL$_b$ was eluted with the V$_e$ of the initial LPL$_a$, and one with the V$_e$ of LPL$_b$. Both of these activities were still markedly suppressed by heparin, and the shape of the second peak remained typically broad (Fig. 7). Similar results were obtained in a second experiment.

Molecular size. LPL$_a$ was eluted from our chromatographic columns with the V$_e$ of totally excluded proteins, this void volume having been determined by measuring the elution volume of tobacco mosaic virus (mol wt 40.7 × 10⁶). Since the 8% agarose gel which we used had a molecular weight exclusion limit for globular species of 1.5 × 10⁶, this latter number presumably represents the lower limit for the size of LPL$_a$. LPL$_b$ was eluted in an intermediate position between two calibrating proteins, bovine thyroglobulin, mol wt 7 × 10⁵, and human γ globulin, mol wt 1.6 × 10⁵. Relating the sizes of the lipases to the protein standards suggests that LPL$_a$ is at
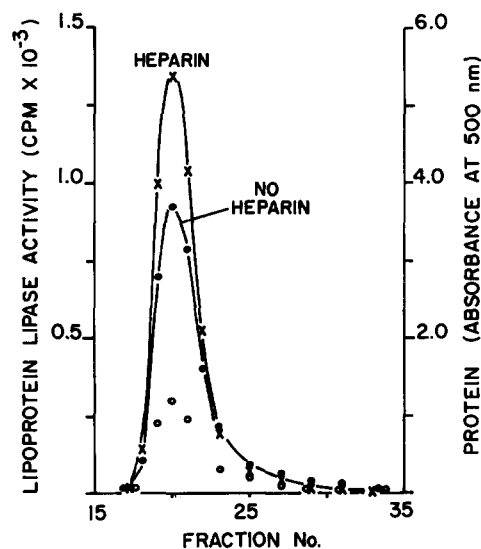

FIG. 6. Effect of heparin on the elution profile of rechromatographed LPL$_a$. NH$_4$OH–NH$_4$Cl extracts of adipose acetone powder were applied to two columns of Bio-Gel A-1.5M (1.5 × 80 and 1.5 × 83 cm). Fractions from the ascending edge of the first activity peaks were combined and concentrated, and this 7.5 mg of protein was applied to a Bio-Gel A-1.5M column (1.5 × 80 cm). 2.0-ml fractions were collected at the rate of 6 ml/hr, the void volume (45 ml) corresponding to fractions 1–19. 0.4-ml aliquots were assayed for lipoprotein lipase activity with (X) and without (●) 1.9 μg of heparin. Results are expressed as cpm/0.4 ml. Protein (○) was determined colorimetrically on 0.2-ml aliquots and is expressed as absorbance units/0.2 ml.
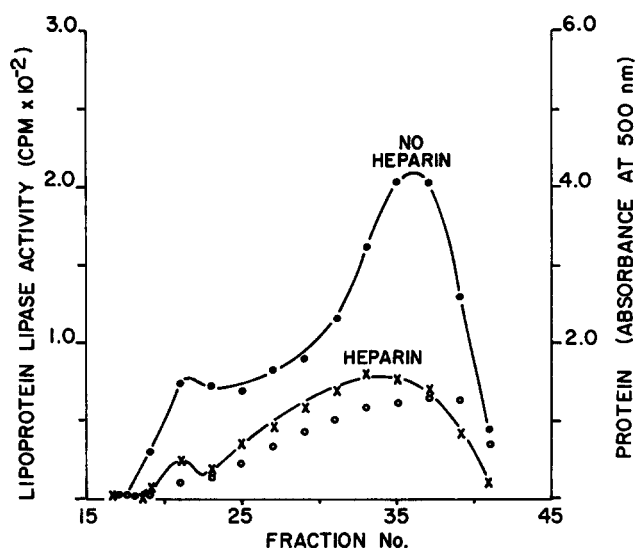
FIG. 7.   Effect of heparin on the elution profile of rechromato-graphed LPL$_b$. NH$_4$OH–NH$_4$Cl extracts of adipose acetone powder were applied to two columns of Bio-Gel A-1.5M (1.5 × 80 and 1.5 × 83 cm). Fractions from the descending edge of the second activity peaks were combined and concentrated, and this 24 mg of protein was applied to a Bio-Gel A-1.5M column (1.5 × 83 cm). 1.7-ml fractions were collected at the rate of 5.1 ml/hr, the void volume (45 ml) corresponding to fractions 1–20. 0.4-ml aliquots were assayed for lipoprotein lipase activity with (X) and without (●) 1.9 μg of heparin. Results are expressed as cpm/0.4 ml. Protein (○) was determined colorimetrically on 0.2-ml aliquots and is expressed as absorbance units/0.2 ml.

least five times the size of LPL$_b$. At this stage of our study we can speak only of the molecular sizes of the lipoprotein lipase species, since we have neither purified the fractions beyond the recycling experiments nor determined whether lipids not removed by acetone–ether still remain associated with the lipase protein.

DISCUSSION

These data show that there are in extracts of rat adipose tissue at least two molecular species exhibiting the classical properties of lipoprotein lipase and differing in size, or possibly shape, and response to heparin. The larger fraction has been designated LPL$_a$ and the smaller LPL$_b$. The LPL$_a$ separated from such extracts showed a 60–100% stimulation by heparin and LPL$_b$ was inhibited by like percentages, the sum of the activation and inhibition closely approximating the total change in the original crude extract. This observation emphasizes the difficulty in interpreting data derived from experiments with crude extracts.

Although the interrelationship of heparin with lipoprotein lipase is not yet understood, it has been intensively investigated. Addition of heparin to lipoprotein lipase from a variety of sources has not produced a uniform response. Employing NH$_4$OH–NH$_4$Cl extracts of adipocyte acetone powder, Patten and Hollenberg

(14) concluded that heparin increased enzyme activity by increasing binding of enzyme to the chylomicron substrate, possibly forming additional binding sites on the enzyme molecule or stimulating, by allosteric activation, the existing binding sites. Whayne and Felts (15) have published data strongly suggesting the existence of an allosteric site for heparin on the molecule of lipoprotein lipase in guinea pig postheparin serum, the heparin functioning as a specific ligand to alter the kinetics of interaction of LPL with its effective substrate. In an investigation of lipoprotein lipase activity in cow's milk, Olivecrona (16) explained that the lack of responsiveness of the cream enzyme to heparin was due to the enzyme being already fully saturated with heparin, whereas the skim milk enzyme, activated by exogenous heparin, was not previously saturated. Similarly, Robinson and Wing (17) have expressed the view that endogenous heparin, or a heparin analog, may be a component of the functional enzyme and also that heparin may "stabilize" the enzyme. Gartner and Vahouny (18), in discussing their differential purification of an "inherent" and "heparin-activated" lipoprotein lipase in rat heart homogenates, support the possibility that heparin may be a prosthetic group for lipoprotein lipase, the inactive apoenzyme requiring heparin for activation. However, all these measurements were made in crude mixtures.

The size of one lipoprotein lipase, LPL$_a$, from rat adipose tissue seems much larger, on the basis of molecular weight, than the enzyme from other sources. However, the size of LPL$_b$ is of the same order of magnitude as the lipoprotein lipase from cow's cream reported by Olivecrona to have a molecular weight corresponding to that of a globular protein of 115,000 (16). Fielding purified a smaller lipoprotein lipase, mol wt 72,000, from rat postheparin plasma (19). It is difficult to relate these observations, since the enzymes were derived from various sources by different methods of preparation.

Our data do not indicate that LPL$_a$ and LPL$_b$ are interconvertible forms beyond the demonstrable aggregation of LPL$_b$ into a larger entity. A number of possible relationships between the two species can be postulated. LPL$_b$ may be a less active precursor or subunit of LPL$_a$; it is synthesized in the cell and becomes "activated" on release into the tissue (20) by the addition of heparin, lipid, or some unknown factor. Alternatively, activation/inactivation of lipoprotein lipase may be due to phosphorylation, i.e., LPL$_a$ may be phosphorylated to LPL$_b$ or vice versa, analogous to the glycogen phosphorylase system.

Another particularly intriguing possibility is that LPL$_a$ and LPL$_b$ are different proteins specified by separate m-RNAs and that LPL$_b$ is a constitutive enzyme, whereas LPL$_a$ is inducible. This last hypothesis could account for

the increase of lipoprotein lipase in adipose tissue in response to glucose and to actinomycin (3, 8) which is presently an enigma.

For some years, in interpreting their studies of lipoprotein lipase, investigators have suggested the occurrence of two forms of the enzyme, but these interpretations have been essentially deductive. Our finding that LPL_a and LPL_b can be separated by gel chromatography may be used to substantiate these studies.

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