Lipoprotein Lipase: Comparative Properties of the Membrane-Supported and Solubilized Enzyme Species†

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ABSTRACT: The catalytic rate of lipoprotein lipase has been determined before and after solubilization from the perfused rat heart. Both the apparent K_m and k_c values were closely similar for the membrane-bound and soluble lipase. This result was confirmed for the major plasma very low density lipoprotein fraction (S_f 100–400) and for two subfractions of rat lymph chylomicrons (S_f 100–400 and S_f >400). These findings suggest a superficial binding site for lipoprotein lipase at the capillary wall, and the absence of major conformational change during solubilization. Both membrane-bound and soluble lipase showed catalytic rates about twofold greater with chylomicron than with very low density lipoprotein triglyceride. These findings are discussed in the light of recent ideas on the mechanism of lipoprotein lipase activity.

In recent years many studies have been made of the kinetic properties of solid-supported enzymes. Many enzymes which are normally membrane-bound have been shown to have changed reaction velocities after solubilization (McLaren and Babcock, 1959). Other studies have been of soluble enzymes covalently linked with solid supports such as substituted celluloses (Lilly *et al.*, 1966; Hornby *et al.*, 1968) or polystyrene (Goldstein, 1971) or trapped within gel slices (Bunting and Laidler, 1972). A number of theoretical treatments of the kinetics of solid-supported enzymes have been published, either for stirred suspensions or for enzyme packed within columns through which substrate percolates (Lilly *et al.*, 1966; Goldman *et al.*, 1971; Sundaram *et al.*, 1970). The activities of such immobilized enzymes in many cases follow Michaelis–Menten kinetics but with significantly changed kinetic constants, often involving an increased apparent Michaelis constant and a much decreased catalytic rate. In the case of charged supports there may be a displacement of the pH–activity profile (Goldstein, 1971).

A small number of reported enzymes including histaminase (Dahlback *et al.*, 1968), lipoprotein lipase (Korn, 1959), and a hepatic lipase (Hamilton, 1964) are solubilized from a membrane binding site by polyanions such as heparin. Lipoprotein lipase has been characterized in soluble form from post-heparin plasma (Fielding, 1969, 1970a,b) and the apparent kinetic constants have been determined for both natural lipoprotein substrates and for synthetic dispersions of neutral lipids (Fielding, 1973). Attempts to obtain an active preparation of membrane-bound lipase have so far been unsuccessful (Rodbell, 1964) and studies on the substrate specificity and mechanism of lipase activity have been limited to the solubilized enzyme. The purpose of the present study has been to compare the properties of the lipoprotein lipase of the rat heart endothelial membrane before and after solubilization by heparin.

Experimental Section

Materials. Animal donors in all experiments were male Sprague-Dawley rats, 300–350 g, fasted overnight before use. [*N-sulfonate*-^{35}S]Heparin was purchased from Amersham-Searle, and unlabeled heparin from Invenex, San Francisco. Albumin (bovine Fraction V) (Reheis), made to 15% w/v in 0.154 M NaCl, was adjusted to pH 7.4 with 1 M NaOH and dialyzed overnight against 500 volumes of 0.154 M NaCl. [1-^{14}C]Palmitic acid and [9,10-^{3}H]palmitic acid (Amersham-Searle) were complexed with albumin as the potassium salts (Felts and Masoro, 1959).

Lipoprotein Preparation. Rat serum very low density lipoprotein labeled in the triglyceride moiety (Table I) was prepared from the blood of rats injected 40 min previously with 50–150 μCi of albumin-palmitate complex through the femoral vein. Lipoproteins were fractionated at 2–4° by ultracentrifugation in the presence of 0.1% disodium ethylenediaminetetraacetic acid (pH 7.4). The lipoprotein subclass of flotation index (S_f)[1] 100–400 was isolated by layering serum, without adjustment of density under an equal volume of Krebs-Ringer (Table I). The 0.61 × 10^6 g-av. min cut (S_f >400) was discarded, and the required fraction was floated by centrifugation for 4.86 × 10^6 g-av. min (Dole and Hamlin, 1962). This was twice washed by recentrifuging under the same conditions. Composition of this fraction is shown in Table I. Further centrifugation did not change this composition. This subfraction contained about 50% of total lipoprotein triglyceride and about the same proportion of lipoprotein radioactivity. Subfractions of rat chylomicrons were prepared from the lymph of animals with a draining mesenteric lymph duct cannula and an inflow cannula to the duodenum through which triglyceride emulsion (Intralipid, Vitrum, Sweden; 2.5% w/v in 0.154 M NaCl, containing 5 μCi of [^{3}H]- or [^{14}C]palmitate-albumin complex/ml) was passed at a flow rate of 2–3 ml/hr. Lymph was collected into ice-cooled tubes and centrifuged at 9.5 × 10^4 g-av. min to remove the largest particles (S_f >5000). The residual chylomicron triglyceride was then fractionated into S_f >400 and 100–400 components as de-

† From the Cardiovascular Research Institute, University of California, San Francisco, California 94143. *Received May 23, 1974.* This work was supported by Grant No. HL 14237 from the U. S. Public Health Service and No. 71-1064 from the American Heart Association. C. J. F. is Established Investigator (70-142) of the American Heart Association.

[1] Abbreviations used are: S_f, flotation index at solvent density 1.063 g/ml (Dole and Hamlin, 1962); k_c, $k_{c'}$, catalytic constants for the soluble and membrane-bound lipase species; K_m, $K_{m'}$, apparent Michaelis constants for the soluble and membrane-bound lipase species.

TABLE I: Lipid and Protein Composition of Lipoprotein Substrates (by weight).[a]

	Very Low Density Lipoproteins (S_f 100–400)	Chylomicrons (S_f 100–400)	Chylomicrons (S_f >400)
Triglyceride	77.5 ± 0.8	78.4 ± 1.1 (78.8 ± 2.1)	84.9 ± 1.0 (85.2 ± 2.4)
Phospholipid	8.7 ± 0.3	18.3 ± 1.1 (16.3 ± 2.6)	13.5 ± 1.1 (10.6 ± 3.0)
Free cholesterol	3.0 ± 0.6	1.0 ± 0.7 (1.4 ± 0.5)	0.4 ± 0.2 (0.7 ± 0.2)
Cholesteryl ester	3.4 ± 0.7	0.8 ± 0.4 (1.2 ± 0.5)	0.3 ± 0.1 (1.4 ± 0.4)
Protein	7.3 ± 0.6	1.8 ± 0.2 (2.2 ± 0.3)	0.9 ± 0.2 (2.0 ± 0.5)

[a] Values are means ± SD of three preparations. Values in parentheses represent composition of the same mesenteric chylomicron preparations preincubated with perfusion medium for 30 min at 37° in the ratio 1 μmol of chylomicron triglyceride/ml of medium, then reisolated by centrifugation as before. Lipid classes were determined as described previously (Fielding *et al.*, 1974).

scribed for very low density lipoprotein. Composition of lipoprotein fractions was also determined after incubation with perfusion medium, then reisolated by centrifugation (Table I).

Perfusion Procedure. Isolated rat hearts were perfused with a modified recirculating Langendorff apparatus (Morgan *et al.*, 1961). Perfusion was carried out using Krebs-Henseleit bicarbonate buffer gassed with 95% O_2–5% CO_2. All glass surfaces were siliconized with Siliclad (Clay-Adams, N. Y.) and under these conditions no radioactivity was lost from labeled lipoprotein fractions to the apparatus.

Concentrated recalcified citrated plasma, free of detectable very low density lipoproteins, was prepared by centrifugation for 24 hr at 40,000 rpm in the 40.3 rotor of the ultracentrifuge. The infranatant fraction was dialyzed against 500 volumes of 0.154 M NaCl, 0.0056 M KCl, 0.0014 M KH_2PO_4, 0.001 M $MgSO_4$, 0.003 M disodium ethylenediaminetetraacetic acid (pH 7.4). The reservoir of the perfusion apparatus contained 5.0 ml of lipoprotein solution, 3.0 ml of dialyzed 15% w/v albumin solution, 0.5 ml of threefold concentrated plasma, 0.1 ml of 0.154 M NaCl, 0.38 ml of 0.154 M $CaCl_2$, 0.03 ml of 0.154 M KH_2PO_4, 0.03 ml of 0.154 M $MgSO_4$, 1.78 ml 0.154 M $NaHCO_3$ (saturated with carbon dioxide), and 0.4 ml of 0.1 M glucose. Perfusion medium was passed through the heart chamber at a flow rate of 8.0–8.5 ml/min. Temperature of the perfusion chamber was 37 ± 0.5°. pH throughout the perfusion period was 7.35–7.40. p_{O_2} entering the heart was 250–330 mm, and p_{O_2} exiting was 100–130 mm. The heart rate was maintained within the range 140–190/min throughout the perfusion period. Under these conditions there was no significant denaturation of substrates in terms of enzyme activity, as described below. Neither heart beat nor triglyceride removal rate was increased by addition of 10% v/v packed washed rat erythrocytes. Volume of the heart space and connecting tubing was 3.0–3.5 ml, as determined with Evans Blue. Total perfusion period was usually 45–60 min during which 2–3 separate reservoirs of different initial lipoprotein concentration were perfused through the heart. Between perfusions the heart was washed with Krebs-Ringer bicarbonate buffer for 2–3 min. During each perfusion period 5–7 duplicate 0.1-ml samples of perfusion medium were withdrawn from the system for analysis of triglyceride radioactivity. Samples were mixed with 0.154 M NaCl (0.1 ml); then with 1:2 v/v chloroform–methanol (0.75 ml); and finally with 0.154 M NaCl (0.25 ml) and chloroform (0.25 ml) to separate the phases. Aliquots of the lower organic phase were taken for thin-layer chromatography on silica gel layers on glass plates (Merck) developed in hexane–diethyl ether–acetic acid, 83:16:1 v/v. The triglyceride band was visualized with iodine vapor, scraped off, and counted by liquid scintillation procedures previously described (Fielding and Fielding, 1971). Recovery of triglyceride through the procedure was 86 ± 3.1% (SD). Triglyceride removal rates were determined following chemical and radiochemical analysis of the lipoprotein fractions. Rates of hydrolysis have been calculated in terms of micromoles of triglyceride removed per minute per milliliter initial recirculating volume.

To identify denaturation of very low density lipoproteins or chylomicrons during perfusion, three-times centrifuged lipoprotein was recirculated through the apparatus for 45 min in the absence of the heart, then mixed with once-spun lipoprotein carrying the alternate label, and the mixture was injected into intact rats. The removal rate for each label was found to be identical, and the isotope ratio during removal did not change significantly from that of the original mixture (Figure 1).

Solubilization of Lipoprotein Lipase. Lipase activity was

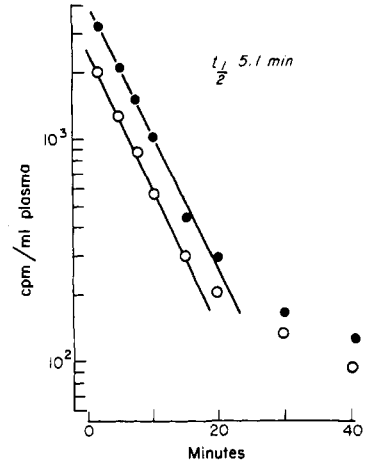

FIGURE 1: Removal of ^3H very low density lipoprotein and preperfused ^{14}C very low density lipoprotein in the intact rat. ^{14}C very low density lipoprotein was perfused through the Langendorff apparatus for 30 min at 0.25 μmol of triglyceride/ml in the absence of the heart, then 0.5 ml of perfusate mixed with 1.0 ml of ^3H very low density lipoprotein (0.17 μmol of triglyceride/ml) and injected into the femoral vein of an intact animal. Blood samples were taken serially from the tail for analysis of serum triglyceride radioactivity.

TABLE II: Effect of Heparin on Post-Heparin Lipase Activity.[a]

Fraction	Lipase Activity (μmol of Triglyceride/min)	[^{35}S]Heparin (μg)
Post-heparin perfusate	0.194	40.0
Dialysate	0.197	33.9
Column eluate		
−heparin	0.063	<0.02
+heparin	0.165	

[a] Lipase activity and heparin content of 1-min post-heparin perfusate in perfusion medium containing 10.0 μg of [^{35}S]-heparin (6.0 μCi mg^{-2}) ml of perfusate^{-1}. Lipase activity was assayed with very low density lipoprotein at pH 7.4 at 37° for 30 min in the presence or absence of added [^{35}S]heparin (10 μg/ml).

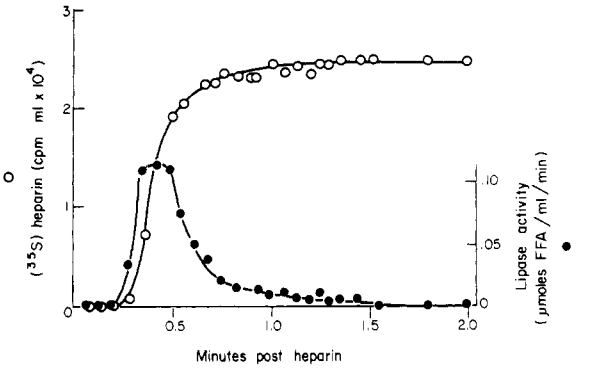

FIGURE 2: Release of post-[^{35}S]heparin lipase from the perfused heart. Perfusion (10 μg of heparin/ml) was at 8.5 ml/min in the presence of 1.0 μmol/ml of chylomicron triglyceride. Lipase activity was determined titrimetrically as release of unesterified fatty acid per ml of perfusate per min in the presence of heparin.

released from the perfused heart by addition of 10 μg/ml of heparin to the assay medium. The effluent was collected without recirculation into ice-cooled tubes. Lipase activity was either assayed directly (in the presence of heparin) or after removal of heparin through an anion-exchange resin. In some experiments both triglyceride removal and production of unesterified fatty acids (assayed titrimetrically with the Radiometer autoburet (Fielding, 1973)) were measured and indicated liberation of 2.1 ± 0.1 mol of fatty acid/mol of triglyceride. To remove heparin, samples of post-heparin perfusion medium (one minute post-heparin) were dialyzed for 3 hr at 0–2° against 500 volumes of 0.1 M sodium phosphate buffer (pH 7.4), then passed through a column of Xerolite FF-IP resin equilibrated with phosphate buffer. Recovery of lipase was about 80%. More than 99.9% of radioactivity was removed from post-[^{35}S]heparin perfusion medium (Table II). The effluent was desalted by passage through a 20 × 2 cm column of Sephadex G-50 gel (Pharmacia) in Krebs-Ringer solution (pH 7.4) and then assayed for lipase activity as described above. Recovery of lipase through the column was also about 80%. Because recovery of lipase by this procedure was not complete, total lipase released from the heart, corrected for stimulation by heparin, was determined directly from the post-heparin effluent. It was also determined by independent measurement of the stimulation of lipase by readdition of heparin to the gel column effluent. Heparin under these conditions increased lipase activity 2.10 ± 0.26-fold (six experiments: mean ± SD). No difference was found between the activities of radioactive and unlabeled heparin in the stimulation of lipase activity.

Determination of Kinetic Constants. The kinetic constants of solubilized lipoprotein lipase in the absence of heparin were determined from the concentration dependence of velocity using very low density lipoprotein or chylomicron substrates. The apparent Michaelis and catalytic constants were determined by least squares from the double reciprocal plots for at least six experimental points. In these experiments the apparent catalytic rate (k_c) was determined from the maximal velocity (V_{max}) as

$$V_{max} = k_c E \quad (1)$$

where E is the total enzyme concentration in moles (Fielding, 1969; Fielding *et al.*, 1974). Specific activity of lipase protein was determined in separate experiments in which the pooled post-heparin perfusate of groups of five hearts was purified as previously described (Fielding, 1969, 1970a). Specific activity was similar to that previously obtained for the lipase purified from whole post-heparin plasma with very low density lipoprotein substrate (Fielding, 1973). E was calculated from the experimental values for this fraction and for chylomicrons and the rate constant (k_c) expressed as moles of substrate consumed per minute per moles of enzyme protein, for the *in vitro* experiments.

The kinetic constants of lipase at the capillary binding site were determined using a modification of the equation of Lilly *et al.* (1966) for reaction of the solid-supported enzyme perfused with substrate within a column. By integration of the Michaelis–Menten equation

$$s_0 - s_t = k_c E t + K_m \ln (s_t/s_0) \quad (2)$$

where s_0 is the initial substrate concentration, s_t the concentration of substrate after time t, E the total enzyme concentration, and K_m the Michaelis constant. When the flow rate of substrate is Q

$$s_0 - s_t - K_{m'} \ln (s_t/s_0) = k_{c'}(E/Q)(v_1/v_t) \quad (3)$$

where v_1, v_t are the void volume and total volume of the enzyme system (such that $Q = v_1/t$) and K_m, $k_{c'}$ are the corresponding kinetic constants of the supported system.

For low substrate concentrations ($s_0/K_m \ll 1$)

$$(s_0 - s_t)/K_{m'} - \ln (s_t/s_0) = k_{c'}(E/Q)(v_1/v_t)/K_{m'} \quad (4)$$

Substituting in Q

$$1/t \ln (s_0/s_t) = k_{c'}E/v_t K_{m'} \quad (5)$$

and the plot of ln (s_0/s_t) *vs.* t has the slope $k_{c'}E/v_t K_{m'}$ where $k_{c'}E/v_t$ is the apparent maximal removal rate of substrate per milliliter column volume. Similarly at high substrate concentration ($s_0/K_m \gg 1$)

$$(s_0 - s_t)/t = k_{c'}E/v_t \quad (6)$$

for enzyme limited to the walls of the column

$$v_1 = v_t \quad (7)$$

Thus, from the slope of the first-order plot at low concentration

$$(\text{eq } 4)\ K_{m'} = k_{c'}E/\text{slope} \quad (8)$$

where $k_{c'}E$ is obtained from the slope of the zero-order plot at high substrate concentration.

The apparent Michaelis constants for membrane-bound

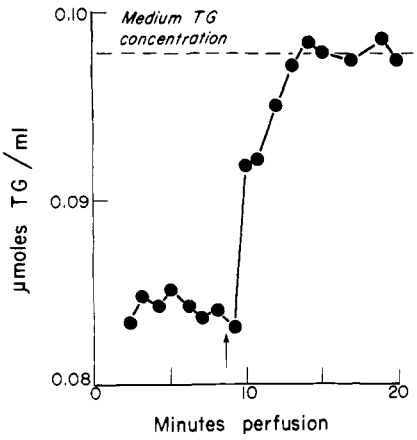

FIGURE 3: Triglyceride hydrolysis in the perfused heart before and after exposure to a constant infusion (10 µg/ml) heparin. Substrate, chylomicron triglyceride (0.1 µmol/ml). Flow rate; 4.2 ml/min. 0.1-ml samples were assayed for triglyceride radioactivity from the heart effluent as described under Methods.

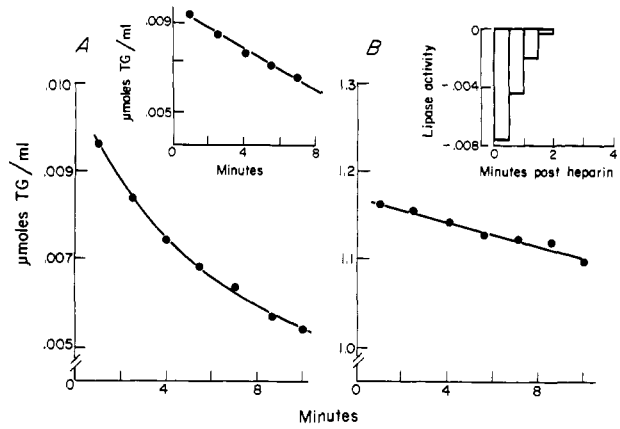

FIGURE 4: Removal of chylomicron lipoprotein triglyceride by the perfused heart at high and low triglyceride concentrations (A) first-order removal rate, initial triglyceride concentration 0.01 µmol/ml. Insert: semi-log plot of triglyceride removal (B) zero-order removal rate, initial triglyceride concentration 1.16 µmol/ml. Insert: lipase activity post-heparin expressed as µmols of triglyceride hydrolyzed per ml of perfusate per min. Flow rate, 8.5 ml/min.

and solubilized lipase (K_m, $K_{m'}$) have been determined for several lipoprotein substrates. Maximal reaction velocities for the lipase of heart endothelium ($V_{m'}$) and for the same lipase solubilized by heparin(V_m) (corrected for the 2.1-fold stimulation induced by heparin) have also been determined. The ratio $k_{c'}/k_c$ was calculated from the k_c directly determined for the soluble enzyme.

Results

Effect of Flow Rate upon Reaction Velocity. At the flow rate used in these experiments (8–8.5 ml/min) $K_{m'}$ was found to be independent of flow rate. This represents the limiting case described by Lilly *et al.* (1966). Under these conditions k_c was also independent of the rate of flow.

Release of Lipase by Heparin. As shown in Figure 2, heparin released lipoprotein lipase activity very rapidly into solution in a single peak whose release was normally 95% complete within 1-min post-heparin. Loss of ability to hydrolyze lipoprotein-triglyceride was also monitored by passing substrate through the apparatus in a nonrecirculating system and determining the loss of triglyceride radioactivity across the heart. As shown in Figure 3, all detectable ability to hydrolyze triglyceride was lost after the passage of heparin. However, uptake of radioactivity from labeled palmitate–albumin was unchanged under the same conditions. In a second series of experiments the zero-order rate of hydrolysis by the perfused heart was correlated with the total lipase activity released by heparin and assayed in the perfusate. A satisfactory correlation ($r = 0.90$) was obtained over a threefold range of heparin-releasable heart lipase activities for a twofold greater activity of soluble lipase, as discussed below.

Triglyceride Hydrolysis during Perfusion. Lipase activity released by heparin was not significantly changed by preperfusion with medium in the presence or absence of high or low concentrations of lipoprotein substrate. In other experiments, the endogenous lipase level was followed by determining the zero-order removal rate for periods up to 90 min. The rate of hydrolysis under these conditions showed no significant change over the period, and was essentially constant by repeated perfusion with fresh medium.

Kinetic Constants of Membrane-Supported Lipase. Rate of triglyceride hydrolysis was zero order within experimental error at concentrations above 0.5–1.0 µmol/ml for both lipoprotein substrates (Figure 4). Similar experiments indicated that substrate removal followed first-order kinetics at circulating triglyceride concentrations <0.01 µmol/ml. The rate of substrate removal was unchanged by a fourfold increase in the concentration of $d > 1.006$ infranatant in the perfusion medium.

Using this procedure $K_{m'}$ and the ratio $V_{m'}/V_m$ were determined for each lipoprotein substrate.

Kinetic Constants of Soluble Lipase. k_c and K_m, the apparent kinetic constants for soluble lipase under the same assay conditions, were determined from the double reciprocal plots obtained for each substrate. Comparison of the values obtained for both sizes of chylomicrons and very low density lipoproteins indicated that the former had a Michaelis constant approximately 1.6-fold greater than the latter ($K_m = 7.5\text{–}8.3 \times 10^{-5}$ M *vs.* $K_m = 5.3 \times 10^{-5}$ M). These values may be compared with the value previously obtained for lipoprotein lipase purified from whole post-heparin plasma under somewhat different experimental conditions (pH 7.0, Tris-HCl buffer) (Fielding, 1973). In this case, K_m for very low density lipoprotein was 2.6×10^{-5} M, and the Michaelis constant for lipase with synthetic lipoproteins prepared by sonication of lipoprotein protein and triolein was about tenfold greater. As shown in Table III, the $K_{m'}/K_m$ ratio for all substrates in the present experiments was close to unity. In the same way, comparison of the calculated ratio of catalytic constants ($k_{c'}/k_c$) for each substrate obtained from $V_{m'}/V_m$ was also close to unity. On the other hand, significantly different catalytic rates were obtained by comparing chylomicron and very low density lipoprotein substrates for both the soluble and matrix-supported lipase species (Table III). In each case chylomicron triglyceride was hydrolyzed at approximately twice the rate of triglyceride present in very low density lipoproteins from plasma. No significant difference was obtained either *in vitro* or in the perfused heart between the hydrolytic rates with the different subfractions of chylomicrons.

Discussion

The lipoprotein lipase system represents the major or only pathway for the supply of triglyceride fatty acids for oxidative or storage purposes in the extrahepatic tissues (Korn, 1959). The properties of the enzyme at its natural

TABLE III: Apparent Kinetic Constants of Membrane-Bound and Solubilized Lipoprotein Lipase.

Substrate	k_c[a] (10^3 min^{-1})	$k_{c'}/k_c$	K_m[a] (mM)	$K_{m'}$ (mM)	$K_{m'}/K_m$
Very low density lipoprotein (S_f 100–400)	1.8 ± 0.15	0.87	0.053 ± 0.007	0.062 ± 0.010	1.17
Chylomicrons (S_f 100–400)	3.42 ± 0.20	1.20	0.075 ± 0.008	0.083 ± 0.007	1.11
Chylomicrons (S_f 400–5000)	3.16 ± 0.20	1.04	0.083 ± 0.012	0.090 ± 0.010	1.08

[a] For lipoprotein lipase in solution, pH 7.4, 37°. k_c values for membrane-bound lipase could not be directly determined in these experiments but can be calculated from columns 1 and 2. Very low density lipoprotein experiments: means ± SD for six experiments. Chylomicron fractions: means ± SD for four experiments. Calculation of k_c, $k_{c'}$ is expressed on the basis of the molecular weight of the stable lipase species (72,600) (Fielding, 1969).

membrane site are therefore of interest. Heart tissue also contains lipase activity which is not releasable by heparin and probably represents an intracellular enzyme species (Borensztajn and Robinson, 1970). It plays at least no immediate role in the hydrolysis of circulating triglyceride.

It has not yet proved possible to isolate a fraction containing only endothelial cell membranes. Collagenase digestion, which has been used for the isolation of adipocytes containing intracellular lipoprotein lipase, gave a total capillary endothelial fraction almost devoid of lipase activity (Rodbell, 1964). Lipoprotein lipase in the perfused heart has been previously shown to hydrolyze lipoprotein triglyceride derived either from the gut (chylomicrons) or from the liver (very low density lipoproteins) (Delcher *et al.*, 1965; Borensztajn and Robinson, 1970). However, the present report appears to represent the first attempt to determine to what extent the kinetic properties of the solubilized lipase reflect those of the membrane-supported enzyme.

The kinetic theory of enzyme activity from solid supports has been the subject of several theoretical treatments. The equation obtained by Lilly *et al.* (1966) for column flow is analogous to the plot of s_0/t, *vs.* $1/t \ln (s_0/s_t)$ derived from the integrated Michaelis–Menten equation (Walker and Schmidt, 1944). This equation has been validated for several enzyme systems (Wilson *et al.*, 1968; Taylor and Swaisgood, 1972). In the case of the perfused rat heart several considerations prevent direct substitution of data into this equation. Firstly, preliminary results indicated that $K_{m'}$(app) was not independent of flow rate at <4 ml/min. Under these conditions the time of residence of substrate within the heart ($t' = v_1/Q$) is about 15 sec and the calculated difference in the substrate concentration across the heart, at saturation, was usually <0.003 μmol of triglyceride. Since the concentration of substrate required for zero-order removal rates was 0.5–1.0 μmol/ml, such a difference for zero-order removal was scarcely within the sensitivity of the method. Secondly, contrary to the system of Lilly *et al.*, in the perfused heart the volume occupied by the enzyme at the capillary walls is negligible compared to the system volume ($v_1 = v_t$). The equations used in this study for the recirculating system have been obtained by integration for the limits where substrate concentration is very low ($s_0/K_m \ll 1$) or high ($s_0/K_m \gg 1$). These give results comparable to those obtained for the soluble enzyme system with rate constants $K_{m'}$ and $k_{c'}$ corresponding to the apparent Michaelis constant and catalytic constant for the soluble lipase. These values can be compared with the constants determined for the soluble lipase.

The major errors involved in the estimation of the kinetic constants of membrane-bound lipoprotein lipase appear to be as follows. (1) The maximal concentration of circulating lipoprotein triglyceride that can be used to establish the zero-order removal rate is limited by the sensitivity of the assay for initial and final concentration (the fatty acid product cannot be assayed because this is partly cleared from the circulating medium into the heart tissue). Thus for $K_m = 6 \times 10^{-5}$ M, when circulating triglyceride is 0.6 μmol/ml, the enzyme is about 91% saturated. Under such conditions, the value of $V_{m'}$ is underestimated by about 10% and $k_{m'}$ is underestimated by the same proportion. (2) The removal of heparin from post-heparin perfusate has been assumed to reverse the changes induced by the presence of polyanion. While there is no direct evidence that this is the case, where the recovery of lipase was nearly complete following the ion-exchange procedure, addition of heparin almost restored the original activity of the post-heparin perfusate. It is likely, therefore, that the correction factor used to account for the stimulation of lipase by heparin is a reasonable one. Stimulation of deheparinized plasma by fresh heparin in these experiments is also similar to the fold stimulation previously obtained with acetone-extracted homogenates of heart tissue (Korn, 1959).

The level of lipase at the capillary wall site has been estimated in these experiments by the recovery of soluble lipase from the heart. Hydrolysis of circulating triglyceride was abolished following passage of heparin, while clearance of unesterified fatty acid was unchanged. Further, heparin-released lipase activity correlated well with triglyceride hydrolysis in the heart, and after allowance for the effect of heparin itself, the hydrolytic rates were nearly equal (Table III). Both $V_{m'}$ and $K_{m'}$ may be underestimated by as much as 15%. However, it is apparent in any case that the values for the apparent kinetic constants of lipase before and after solubilization were closely similar (Table III).

There are several reasons why solid-supported enzymes would be expected to show different rate constants from those of the soluble form of the same enzyme. The configuration of the enzyme reaction site might be changed by attachment to the matrix (changing K_m, k_c). Also, the local environment of the enzyme site might not be the same as that of the bulk solution, particularly in the case of a charged support (Wilson *et al.*, 1968; Goldstein, 1972). This would be expected to lead to changes in K_m and k_c. Furthermore, if reaction at the binding site was diffusion controlled, $K_{m'}$ (but not k_c) would be increased by a factor (F) related to the diffusion coefficient of the substrate, the enzyme catalytic rate, and the distance of the enzyme from the effective membrane surface (inclusive of the existence of any "unstirred layer") (Sundaram *et al.*, 1970). However, if, as is likely, K_m is not a pure equilibrium constant,

each of these factors might be expected to induce changes in both K_m and k_c. In fact, in the enzyme systems studied so far, attachment to a solid support has been generally shown to give rise to a several-fold change in the kinetic constants of the reaction.

However, the lipoprotein lipase reaction differs in several respects from hydrolase systems previously reported. Firstly, the substrate lipoproteins are particles of very large size, with diameters in the range 50–75 nm for particles of S_f 100–400, and mostly above 100 nm for those of S_f >400 (review; Skipski, 1972). Such particles might induce steric hindrance between adjacent enzyme sites. However, calculation easily shows that when the K_m(app) for chylomicron and very low density lipoprotein substrates is expressed in terms of the maximal cross-sectional area of the particles present per milliliter at the half-saturating concentration of triglyceride for each substrate, this value is actually much less for chylomicrons than for very low density lipoproteins, because of the increased volume/diameter ratio of the larger particles. Also, as shown in Table III, there is, in any case, no significant difference between larger and smaller chylomicrons in terms of kinetic constants, when these are calculated in terms of bulk substrate concentrations. It is therefore unlikely that steric hindrance between substrate particles plays a role in the regulation of lipase activity at the capillary wall. The differences between the compositions shown in Table I include a lower concentration of cholesteryl esters in particles of gut origin. While other evidence indicates that the cholesteryl ester/triglyceride ratio may be important in the determination of the catalytic rate of lipoprotein lipase (Ontko and Vessby, 1973; Fielding and Higgins, 1974) other possibly significant differences include the polypeptide composition of the protein moiety (Kostner and Holasek, 1972), and the free cholesterol/phospholipid ratio (Fielding, 1970b).

Secondly, the catalytic rate for lipase with both lipoprotein and synthetic lipid substrates is quite low (Fielding, 1973). Since k_c appears in the relation of the diffusion-dependence of $K_{m'}$ (Sundaram *et al.*, 1970), the reaction would not be diffusion controlled unless the distance of lipase from the effective membrane surface was large. This is unlikely to be the case because the enzyme is very rapidly released into the circulating perfusion medium. Lipase activity is therefore probably not diffusion limited in the perfused heart under the experimental conditions described.

Although the strong polyanion heparin solubilizes lipoprotein lipase, the purified lipase was unreactive with either soluble or matrix-supported heparin (Fielding *et al.*, 1974). Also, after removal of heparin from post-heparin plasma with an anion-exchange resin column (Table III) the kinetic constants of solubilized lipase showed no major differences from those of the lipase at the membrane site. The absence of such differences most probably results from an absence of major conformational changes at the enzyme active site as a result of release into the medium. It consequently seems unlikely that a "polyanion-binding site" regulates the expression of lipase activity with lipoprotein substrates at the capillary wall. Alternatively, the steric requirements for binding to the membrane site may be thought of as being less stringent than for reaction at the substrate lipoprotein surface. Other evidence for this interpretation might be the tenfold higher apparent K_m for lipase activity with synthetic lipoprotein substrates than with the natural substrates of the enzyme.

Consequently, the results reported here indicate the feasibility of utilizing solubilized lipoprotein lipase in the study of the kinetics of triglyceride removal by this enzyme system. This conclusion could be of value in the study of lipase activity of organs or species where organ perfusion techniques are not practicable. It also suggests that in some circumstances information can be obtained using perfusion procedures to investigate the properties of enzymes bound to the endothelial membrane, in circumstances where enzyme lability might preclude isolation of an active enzyme–membrane complex.

Acknowledgments

We express our appreciation to Mr. M. Bauza, Miss M. Moeller, and Mrs. D. Sock for expert technical assistance in this research.

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Specific Ion Effects in Affinity Chromatography. The Case of L-Histidinol-phosphate Aminotransferase†

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ABSTRACT: Specific ion effects provide an additional parameter which can be adjusted for optimizing the purification of a protein by affinity chromatography. This principle is illustrated in the case of L-histidinol-phosphate aminotransferase (EC 2.6.1.9) from *Salmonella typhimurium,* which was purified 15-fold in one step by chromatography on L-histidinol phosphate coated agarose. The choice of ions in the buffer and the adjustment of their concentration dramatically affect the elution pattern of the column. A 0.7 M Na_2HPO_4 buffer adjusted to pH 7 with citric acid promotes adsorption of the enzyme onto the column, while subsequent dilution of the same buffer results in selective desorption.

The unique affinity of an enzyme for its substrates, inhibitors, cofactors, or other physiological effectors provides a variety of specific interactions which can be used in the design of efficient columns for enzyme purification (Lerman, 1953; Arsenis and McCormick, 1964, 1966; McCormick, 1965; Cuatrecasas *et al.,* 1968; Wilchek and Gorecki, 1969). Initially applied to only a few enzymes, this approach is now extensively used due to the introduction of simple, widely applicable procedures for the activation of beaded agarose (Axen *et al.,* 1967; Porath *et al.,* 1967) and for binding to it a large variety of ligands (Cuatrecasas, 1970; Feinstein, 1971; Cuatrecasas and Anfinsen, 1972).

The choice of the solid matrix, the ligand, and the mode of binding of the ligand to the matrix were all shown to be highly important for achieving efficient and selective retention of a desired protein. Ideally, the desorption of bound protein would be mildest and most efficient if a high affinity substrate analog is used. Very often, however, this is not feasible, either because such a compound is not available, or because it is prohibitively expensive. In most cases, elution of the protein from the column is performed by altering the pH of the buffer, changing its ionic strength, or by using a protein denaturant such as guanidine hydrochloride or urea (Cuatrecases and Anfinsen, 1972).

Several years ago, while studying the process of resolution of glycogen phosphorylase, it was shown that specific ions may act as "deforming agents," *i.e.,* loosen or tighten the structure of presumably unique sites in proteins (Shaltiel *et al.,* 1966). In principle, such specific ion effects could be utilized for optimizing retention and elution of proteins during affinity chromatography. In fact, the possible use of such specific ion effects in protein chromatography was recently shown in the desorption of glycogen phosphorylase from a hydrophobic chromatography column (Er-el *et al.,* 1972; Shaltiel and Er-el, 1973).

This paper illustrates the use of specific ion effects both for achieving retention of an otherwise excluded enzyme and for its subsequent desorption from an affinity chromatography column. The enzyme used, L-histidinol-phosphate aminotransferase (EC 2.6.1.9), catalyzes an essential step (the conversion of imidazoleacetol phosphate to L-histidinol phosphate) in the biosynthesis of L-histidine (Ames *et al.,* 1967), and was recently purified from *Salmonella typhimurium* in a crystalline homogeneous form (Henderson and Snell, 1973).

Studies on the effect of various salts on the activity of the enzyme had shown (G. B. Henderson and E. E. Snell, manuscript in preparation) that it is inhibited by polyvalent ions, such as phosphate or sulfate, and that these ions affect the rate of removal of pyridoxal phosphate from the enzyme, suggesting that phosphate and sulfate have a pronounced effect on the structure of the enzyme.

Materials and Methods

Preparation of Cell Extracts. The histidine auxotroph *S. typhimurium hisE11* was used as a source of L-histidinephosphate aminotransferase. The cells were grown and disrupted as described previously (Henderson and Snell, 1973), and the resulting homogenate was dialyzed against a buffer composed of sodium acetate (25 mM) and 2-mercaptoethanol (25 mM), adjusted to pH 5.0 with HCl. The precipitate was removed by centrifugation for 60 min at 30,000*g* (4°). The clarified protein solution was adjusted to pH 7.5 with solid Tris and stored at 4°.

Crystalline L-histidinol-phosphate aminotransferase (for reference purposes) was prepared by the method of

† From the Department of Biochemistry, University of California, Berkeley California 94720. *Received August 14, 1973.* Supported in part by Grants AM 01448 and AI 01575 from the U. S. Public Health Service.

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