Mechanism of salt-mediated inhibition of lipoprotein lipase

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Abstract The activity of lipoprotein lipase isolated from rat postheparin plasma has been determined with synthetic lipids, in the presence and absence of apoprotein of the natural substrate very low density lipoprotein, as a function of medium ion-pair concentration of a number of different inorganic salts. The several kinetic effects of lipoprotein protein on lipase activity were specifically and quantitatively reversed in the presence of molar sodium chloride or solutions of equivalent effective ion concentrations of other salts. Salt-mediated inhibition was fully reversible by dilution and was independent of substrate concentration. Inhibition was a function of the identity of the salt anion within a Hofmeister (lyotropic) series: $I^- > SCN^- > NO_3^- > Cl^- > F^-$, and, in these terms, was not significantly different for a series of inorganic chlorides (Li^+, Na^+, K^+, Cs^+). The effects of salts on the natural lipoprotein substrates, chylomicrons, and very low density lipoproteins were similar to those obtained with a synthetic lipid–protein substrate complex. These findings are discussed in the light of recent ideas on the activation of lipoprotein lipase.

Supplementary key words medium ion-pair activity · apolipoprotein

Lipoprotein lipase (LPL) hydrolyzes the triglyceride moiety of the chylomicrons and very low density lipoproteins of plasma. The triglyceride hydrolase activity of LPL with synthetic triglyceride substrates is serum-dependent and inhibited by molar sodium chloride (1) and these properties have been used to distinguish LPL activity from the activities of other lipases in plasma (2,3). Their biochemical basis is therefore of considerable interest. It is known that LPL activation by serum is mediated through a specific protein subunit of the natural lipoprotein substrates (4–7), perhaps with other lipoprotein peptides playing a modifying role (7, 8). On the other hand, the inhibition of enzyme activities by strong salt solutions is not a unique or even unusual property in other enzyme systems and detailed analysis of the mechanism of ion-dependence has been made in a number of cases (9–11). However, this report suggests there is a close relationship between these properties in the case of LPL.

METHODS

Preparation of lipoprotein lipase

Lipoprotein lipase was isolated from the plasma of male Sprague-Dawley rats (400–500 g body weight) as previously described (12). Specifically, blood was collected from the aortas of animals injected 3–5 min previously with heparin (Invenex, San Francisco, Cal.) (100 IU/kg body weight). Plasma lipoprotein lipase activity was separated from the bulk of contaminating plasma protein as follows. The plasma was incubated with one-fortieth volume of Intralipid 20% (a synthetic triglyceride–lecithin emulsion, the gift of Vitrum Company, Stockholm, Sweden), and then the enzyme–substrate complex was layered under an equal volume of 10% sucrose (w/v) in 0.05 M NH_4OH–NH_4Cl buffer, pH 8.3. Isolation of the floating lipid–enzyme complex was accomplished by preparative centrifugation using the Spinco L3-50 ultracentrifuge at 4–5°C and the 30-rotor (Beckman Instruments, Spinco Div., Palo Alto, Cal.). The floating enzyme fraction was relayered and isolated again under the same conditions. The procedure was repeated until the enzyme fraction was of constant specific activity (normally 4–5 flotation steps). The lipid–lipase complex was mixed with an equal volume of 0.05 M ammonia buffer containing 0.5% (w/v) sodium deoxycholate and 0.5 mM potassium linolenate. The LPL activity was quantitatively released into solution. After centrifugation for 60 min at 4°C and 25,000 rpm in a swinging-bucket rotor the tubes were pierced to separate the purified enzyme solution from precipitated protein and floating lipid (which still contained significant non-enzyme protein). The solution was delipidated with acetone and ether as previously described (12) and the product was redissolved in ammonia–deoxycholate–linolenate buffer. The solution (contain-

Abbreviations: LPL, lipoprotein lipase; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TMU, tetramethylurea.

ing 20–40 μg of protein/ml) was chromatographed on calcium phosphate. Enzyme activity was completely retained in the presence of this buffer solution. The absorbent was washed with 0.2 M sodium oxalate in ammonia–deoxycholate–linolenate buffer, and then eluted with 0.05 M sodium citrate in ammonia–linolenate buffer.

The product, purified about 4000-fold from plasma, was recovered, in a yield of about 20% from the original postheparin plasma lipolytic activity. Protein was present as a single migrating band, as previously illustrated (12). Storage of enzyme activity at 4°C was associated with the loss of 50% of activity in 12 hr and the generation of high molecular weight material retained at the gel origin. Amino acid and carbohydrate analyses of the product have been reported elsewhere (12).

Other evidence for the homogeneity of the enzyme product includes that obtained by column chromatography on Sephadex G-150 (13), analytical ultracentrifugation (12) and immunoprecipitation using an antibody raised against the purified enzyme protein in rabbits (12). Enzyme activity was eluted with constant specific activity from Sephadex G-150. The presence of a single protein species was confirmed by the linear van Holde–Baldwin plot obtained by analytical ultracentrifugation and by a single precipitin line that was obtained from the reaction of antibody with the purified lipase protein by Ouchterlony immunodiffusion. The absence of apoB protein was confirmed by the lack of reaction with an antibody of high titer raised against rat LDL (12). The absence of detectable levels of apoC polypeptides was shown by the lack of observable migrating material on electrophoresis in the absence of sodium dodecyl sulfate, even on heavily loaded gels, by the absence of reaction with anti HDL antibody, and by finding the expected Michaelis–Menten kinetics for activation by addition of pure human apoC-2 polypeptide (7).

The low level of activity of the purified enzyme with synthetic lipids in the absence of added apolipoprotein was shown to be dependent on the hydrogen-ion concentration in the assay medium (14). Under standard conditions (2 μmoles of triglyceride/ml; pH 8.3) activation by apoprotein was about 20-fold. In comparative experiments the basal activity of LPL species isolated from postheparin plasma and bovine milk were not significantly different (7). Purification of this LPL species from the postheparin perfusate of the isolated perfused rat heart (whose solubilization was associated with complete loss of functional triglyceride hydrolysis by the tissue) identified the LPL activity purified by this procedure as the lipase species active in the catabolism of circulating lipoprotein by the perfused heart (15).

Preparation of lipoproteins

Chylomicrons were prepared from the lymph of animals bearing a cannula in the mesenteric lymph duct and an inflow into the duodenum through which was passed triglyceride dispersed with lecithin (Intralipid) diluted to 20 mg of triglyceride/ml (15) with physiological saline. Lymph was collected in ice-cooled tubes. The largest particles and non-chylomicron proteins were removed by centrifugation at 4–5° using the 40-rotor of the Spinco ultracentrifuge. Two ml of lymph (containing 20–40 mg of triglyceride) were layered under 8 ml of physiological saline and centrifuged for 10 min at 12,500 rpm. After removal of the top 1.0 ml of material (which was replaced with saline) the lymph was further centrifuged for 30 min at 17,500 rpm under the same conditions. The floating product was centrifuged again under the same conditions and the product (containing about half the original triglyceride) was used immediately in the assay of LPL as described below.

Very low density lipoprotein was isolated from plasma of animals by ultracentrifugation in the presence of 0.1% disodium-EDTA pH 7.4 as described by Havel, Eder, and Bragdon (16). The floating lipoprotein layer, obtained after centrifugation at 40,000 rpm for 24 hr at 4°C in the Spinco 40-rotor, was reisolated by a second centrifugation under the same conditions for use in the LPL assay medium.

Very low density lipoprotein was either used directly as a substrate for LPL or was delipidated with ethanol–diethyl ether 2/1 (v/v) at −20°C, washed with cold diethyl ether and then the solvent was removed under nitrogen at 0°C. The delipidated lipoprotein protein (apoVLDL), representing >96% of original lipoprotein protein as assayed by the Lowry reaction (17), was totally soluble at a concentration of 1 mg/ml in 0.05 M NH_4OH–NH_4Cl buffer, pH 8.3, when the solution contained 0.5 mM potassium linolenate and 8 M urea. The solution containing urea and linolenate was dialyzed twice at 4°C against 1000 volumes of 0.05 M NH_4OH–NH_4Cl buffer using Spectrophor dialysis tubing (Spectrum Industries, Los Angeles, Calif., nominal cutoff M.W. 3500) which had been boiled in 1% sodium bicarbonate, 0.1% disodium EDTA and soaked overnight in 8 M urea solution. The dialyzed protein solution was stored at −70°C. The solubilized protein (which contained the whole of the original protein precipitate) retained cofactor activity in the LPL assay (as μg

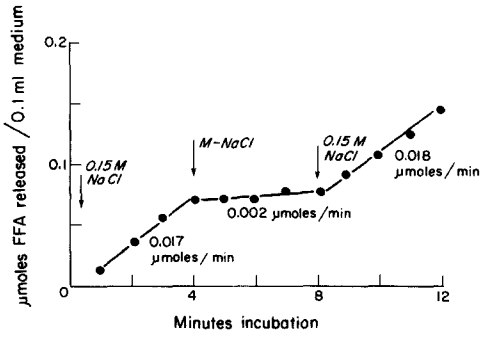

Fig. 1. Hydrolysis of triolein + apoVLDL in the presence and absence of 1 M NaCl. 0.6 ml of purified LPL was mixed with 3.4 ml of assay medium (pH 8.3) containing NaCl to give a final concentration of 0.15 M. During incubation at 37°C duplicate 0.1 ml samples were taken for analysis of unesterified fatty acid at zero time and at one minute intervals thereafter. After 4 min, 2.8 ml of remaining medium were mixed with 0.7 ml of assay medium containing 4.4 M NaCl to a final concentration of 1 M NaCl. Duplicate 0.1 ml samples were taken at zero time and at one minute intervals for 4 min. The residual medium (2.3 ml) was then mixed with 5.7 volumes (13.1 ml) of assay medium made up in distilled water. Duplicate 1.0 ml assays were taken at zero time and at each minute thereafter for four minutes. Means of duplicate assays are shown, corrected to the original assay volume to permit comparison between initial and final rates. Rates were calculated from the experimental points fitted by least squares.

of apoVLDL required for half-maximal activation) (7) unchanged for at least 12 months.

Synthetic lipoprotein complexes and dispersed lipid substrates were prepared by sonic irradiation using a Branson Sonifier (Heat Systems, Plainview, N.Y.) with the standard probe. Triolein (Sigma Chemical Corp., St. Louis, Mo.) (more than 99% pure by chromatography in the laboratory) (14) in heptane solution, was mixed with 10 moles % of synthetic L-α-dioleyl lecithin (Serdary, London, Ontario) in ether solution, and then dried under nitrogen. Sonication was carried out in 3–4 ml lots in 10-ml glass vessels. Distilled water was added to give a concentration of 10 μmoles of neutral glyceride/ml. Sonication was for 2 one-min periods. Initial temperature was 37°C and the final temperature was less than 42°C. Nominal tip power was 60–70 watts. If detectable floating lipid remained, the preparation was discarded. In some experiments, as specified, dispersion was in the presence of apoVLDL at a concentration of 5 μg of protein/μmole of lipid, which is saturating for this system (14).

Adsorption of apoVLDL to lipid was confirmed by flotation of the lipoprotein complex in 0.15–1.5 M NaCl. One ml of sample in salt solution was made to 5% with sucrose and then layered under the same volume of the corresponding salt solution in the 2-ml adaptor tubes of the Spinco 40.3 rotor. Flotation was for 30 min at 30,000 rpm. The upper floating layer was recovered by slicing, and aliquots of both supernatant and infranatant fractions were assayed for protein content (17).

The proportion of total protein of VLDL, apoVLDL, or synthetic lipoprotein complexes that was soluble in N,N,N′,N′-tetramethylurea was determined by mixing protein solutions (containing about 100 μg protein/ml) with an equal volume of tetramethylurea, followed by incubation for 30 min at 37°C (18, 19). Precipitated protein was removed by centrifugation in the ultracentrifuge at 10,000 rpm for 20 min, then the soluble fraction was dialyzed against 500 volumes of 0.15 M NaCl, using washed pretreated dialysis tubing prepared as described above. Protein content of aliquots of the original lipoprotein preparations and tetramethylurea-soluble protein were determined by a micromodification of the procedure of Lowry et al. previously described (13).

Assay of enzyme activity

LPL was assayed as release of fatty acid at 37°C in the presence of lipid or lipoprotein substrates (0.25–3.0 μmoles/ml of neutral glyceride), albumin (final concentration 2% by weight) (bovine Fraction V, Reheis, Chicago, Ill.) brought to the required hydrogen-ion concentration with 1 M NaOH, and inorganic salts with a final concentration of 0.1–2.0 M (14). The incubation period was usually 10 min and duplicate or triplicate 1 ml samples were taken for assay of fatty acid content at zero time and time points during the assay. Hydrogen ion concentration was maintained ±0.1 pH unit during the period of assay. Reaction was initiated by addition of 10 μl of purified lipase (containing 10–50 μg of LPL/ml) per ml of assay medium and was linear for at least 10 min in the presence or absence of 1 M NaCl (**Fig. 1**). Titration of fatty acids was with 0.06 mM tetramethylammonium hydroxide, using a Radiometer ABU 11 automatic titrating unit. Reproducibility of titrant volume for neutrality with phenol red indicator was ±3 μl, equivalent to $\pm 2 \times 10^{-9}$ moles of fatty acid. Maximal reaction velocity (V_{max}) and apparent Michaelis constant $K_{m(app)}$ were determined by least squares for at least five experimental points using a desk-top computer. Ion-pair activities were calculated using activity coefficients obtained from the standard tables (20).

Phospholipase activity of isolated LPL with triolein–lecithin substrate was determined chemically after incubation at pH 7.0 in the same assay medium as described above. 0.2-ml samples were withdrawn at zero time, and at intervals thereafter, and mixed either with 0.75 ml of chloroform–methanol 1:2 (v/v), for assay of neutral lipids or phospholipids,

TABLE 1. Percent TMU-insoluble protein of natural and synthetic lipoprotein species

	Native VLDL	ApoVLDL	ApoVLDL-TG Complex[a] (0.15M NaCl)	ApoVLDL-TG Complex (1.0M NaCl)
Mean	28.9	26.6	27.2	27.1
SD	(±3.1)	(±2.4)	(±2.6)	(±2.5)

The number of experiments in each case was four. TG complex was prepared by sonic irradiation from triolein and dioleyl lecithin (10 mole %) and apoVLDL (5 μg/μmole triolein). Percent insoluble protein was determined from the protein contents of intact and tetramethylurea-treated lipoproteins as [(total protein − TMU-soluble protein)/total protein].

[a] TG, triglyceride.

or with 25 volumes of heptane–*iso*propanol–2N sulfuric acid 40:10:1 (v/v), for assay of unesterified fatty acids. Samples for phospholipid analysis were centrifuged to precipitate protein, then the soluble extract was dried under N_2, redissolved in 0.2 ml of methanol, and chromatographed on silica gel layers on glass plates developed in chloroform–methanol–water 65:35:4 (v/v). Chromatography was carried out in the presence of egg lecithin and synthetic 1-palmitoyl-lysolecithin standards. Areas of these lipids were dispersed in 5 ml of methanol and aliquots taken for assay of phospholipid as inorganic phosphate by a micromodification (total volume 0.5 ml) of the method of Bartlett (21). Recovery of lecithin through the procedure was 88% and of lysolecithin 86%.

For assay of triglyceride the chloroform–methanol extract was mixed with 0.25 ml of chloroform and the same volume of 0.15 M NaCl and, after mixing and centrifugation to separate the phases, 0.1 ml samples of the lower phase were developed by thin-layer chromatography on silica gel with hexane–diethyl ether–acetic acid 83:16:1 (v/v). Triglyceride-containing areas (and triolein standards) were extracted with chloroform–methanol 1:1, and the glyceride content of the extract was determined by a micromodification of the method of Carlson (22). Recovery of triglyceride through the procedure was complete.

For assay of unesterified fatty acids, heptane (3 ml) and water (2 ml) were added to the acidified organic extract, and the fatty acid content of the heptane phase was assayed by titration as described for the triglyceride hydrolase assay.

RESULTS

Protein content of native and synthetic lipoproteins

As previously reported (14) apoVLDL apoprotein, prepared by delipidation and solubilized with potassium linolenate, was totally complexed with lipid particles prepared by sonic irradiation using the procedure described above. In the present experiments, apoVLDL, prepared by delipidation and containing 98% of original (VLDL) protein content, was complexed with triglyceride–lecithin and flotation was carried out in the presence of 0.15 M or 1.0 M NaCl. Recovery of protein in the floating lipid layer was 98 ± 6% in 0.15 M NaCl and 103 ± 3% in 1 M NaCl (means ± SD, four experiments).

The proportion of tetramethylurea-insoluble protein in intact and delipidated VLDL, and in protein complexed to lipid after flotation in high or low concentrations of salt, was determined. As shown in **Table 1** there was no significant difference in proportions in the different protein preparations, indicating no significant loss of β-protein content from the synthetic lipoprotein species (18).

Basal activity of purified LPL with neutral glycerides

LPL activity with synthetic triglyceride dispersions was low in the absence of apoVLDL (**Table 2**). The

TABLE 2. Effect of NaCl concentration on LPL activity with synthetic and natural lipoprotein substrates

Substrate	Intralipid		Triolein–lecithin		Very low density lipoprotein[a]		Chylomicrons[a]	
pH	7.0	8.3	7.0	8.3	7.0	8.3	7.0	8.3
−apo VLDL (0.15 M NaCl)	0.045	0.092	0.074	0.044				
+apo VLDL (0.15 M NaCl)	0.135	0.343	0.170	0.319	0.110	0.176	0.154	0.335
+apo VLDL (1.0 M NaCl)	0.048	0.088	0.078	0.053	0.060	0.033	0.076	0.073

Values represent μmoles unesterified fatty acid released per 10 min at 37°C from duplicate 1-ml assays containing an initial triglyceride concentration of 3.0 μmoles/ml. NaCl values represent the final salt concentration in the assay medium. Triolein–lecithin synthetic substrates were prepared in the presence or absence of 5 μg apoVLDL/μmole neutral glyceride.

[a] Values are for the intact lipoprotein in the presence of 0.15 M or 1.0 M NaCl.

TABLE 3. Products of LPL activity with triolein-lecithin substrate

Period of Incubation	TG	Lec	LL	FFA	FFA/TG
min	*μmoles/ml assay medium*				
0	1.94	0.14	0.006	0.06	
5	1.73	0.14	0.006	0.48	2.00
10	1.50	0.14	0.007	0.88	2.10
15	1.34	0.14	0.013	1.21	1.91
20	1.23	0.14	0.014	1.43	1.93

Assay was at 37°C at pH 7.0 in the presence of triolein substrate dispersed with egg lecithin. Lipid products of hydrolysis were separated by thin-layer chromatography as described under Methods and estimated microchemically. TG, triglyceride; lec, lecithin; LL, lysolecithin; FFA, unesterified fatty acid; FFA/TG, ratio of unesterified fatty acid produced to triglyceride consumed.

degree of activation by apoVLDL was pH-dependent, but addition of 1 M NaCl to the activated system (+apoVLDL) in each case reduced the rate of lipolysis to that characteristic of the nonactivated system (−apoVLDL) under the same conditions of pH and substrate concentration. Activation by apoVLDL (and inhibition of the activated system by 1 M NaCl) was about 3-fold at pH 7.0 and about 8-fold at pH 8.3 for a triglyceride concentration of 3.0 μmoles/ml. As shown in Table 2 the extent of inhibition of LPL activity with chylomicron and very low density lipoprotein substrates was similar to that found for the synthetic triolein–apoVLDL complex and for Intralipid–apoVLDL. Phospholipase activity, reported for some LPL preparations (23) could contribute to the basal (nonactivated) activity of LPL with triolein–lecithin but not with diolein dispersions. It has been shown that the basal activities with both substrates were similar (14). Analysis of phospholipids in extracts of triolein–lecithin mixtures at zero time and after reaction with LPL indicated only a minor increase in lysolecithin at the end of the incubation period (**Table 3**).

TABLE 4. Flotation of LPL—substrate complex as a function of medium salt concentration

Salt Concentration (NaCl)	Enzyme Recovery Supernatant	Infranatant	Total
M			
0.15	0.97 ± 0.04	0.05 ± 0.02	1.02 ± 0.01
0.50	0.97 ± 0.07	0.03 ± 0.03	1.00 ± 0.07
1.0	0.92 ± 0.08	0.03 ± 0.05	0.95 ± 0.05
1.5	0.90 ± 0.05	0.04 ± 0.04	0.94 ± 0.04

Values are means ± SD for three experiments. Enzyme was complexed with triolein–lecithin substrate in assay medium by preincubation for 3 min at 37°C, then chilled in ice water and brought to the required salt concentration by addition of 5 M NaCl. 1 ml of enzyme solution was layered under the same volume of the corresponding molarity salt solution and centrifuged for 30 min at 30,000 rpm using the 2 ml adaptors of the 40.3 rotor. The floating lipid layer was recovered by slicing and was assayed as described under Methods.

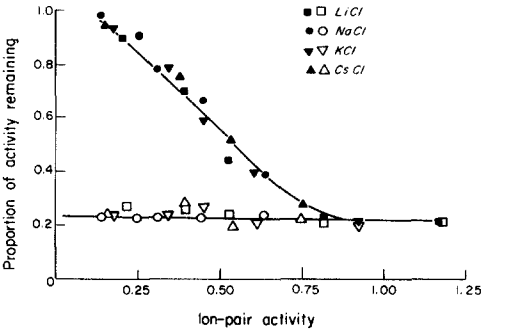

Fig. 2. Inhibition of LPL activity by inorganic chlorides. Maximal reaction velocities were calculated for reaction at medium triolein concentrations of 0.25, 0.5, 0.75, 1.0, and 1.5 μmoles/ml at pH 7.0 for duplicate incubations in each case in the presence and absence of apoVLDL (5 μg/μmole triolein). Open symbols: −apoVLDL, closed symbols: +apoVLDL.

Reversibility of activation and inhibition

As shown in Fig. 1, LPL activity was inhibited by 1 M NaCl almost instantaneously. Inhibition was fully reversible by dilution with salt-free medium. As shown in **Table 4,** when the lipid substrate was isolated by flotation in salt solutions containing 0.15–1.5 M NaCl, recovery of enzyme, which was essentially complete, was almost exclusively in the floating lipid layer. Consequently, the effect of NaCl was to (reversibly) inhibit LPL activity, not to dissociate the preformed enzyme–substrate complex. Salt-mediated inhibition was noncompetitive with respect to triglyceride (**Table 5**) and hence $K_{m(app)}$ was unchanged by the addition of salts under these conditions.

Inhibition of activity by different salts

Inhibition of the rate of the LPL reaction was determined for a series of inorganic salts in terms of the medium ion-pair activity. As shown in **Fig. 2,** when inhibition was mediated by a series of inorganic chlorides there was a significant decrease in V_{max} for reaction of LPL in the presence of apoVLDL. However, in the absence of apoVLDL there was no detectable effect of salt on LPL activity. The regression of all nonactivated rates vs. ion-pair activity shown in Fig. 2 had a slope of −0.02, which was not significantly different from zero. Basal (nonactivated) rates under these conditions were about one-quarter of the activated rates. When medium salt concentrations were calculated in terms of ion-pair activities (20) there was no difference in the abilities of LiCl, NaCl, KCl, and CsCl to inhibit LPL activity.

Similar studies were carried out with a series of potassium salts, including SCN^- and I^- (which have

TABLE 5. Substrate dependence of NaCl-mediated inhibition

Substrate Conc.	Reaction Velocity 0.15 M	Reaction Velocity 1.0 M	Inhibition
mM	*μmoles/ml/10 min*		%
0.25	0.046	0.015	67
0.50	0.073	0.022	70
0.75	0.085	0.030	65
1.00	0.097	0.034	65
1.50	0.117	0.042	64

Reaction velocities are computed slopes from duplicate assays incubated at 37°C for 10 min in the presence of triolein substrate (+10% dioleyl lecithin) and apoVLDL (5 μg/μmole of glyceride) as described under Methods.

major effects on the protein water shell) and F^- (which is poorly polarizable and has little extrinsic effect) (24, 25). As shown in **Fig. 3** these salts had distinct effects on V_{max} for LPL activity in the presence of apoVLDL. Half-maximal inhibition was obtained at salt concentrations of from 0.3 M (KI) to 1.0 M (KF). Like NaCl, these salts were without effect on LPL activity in the absence of apoVLDL. Salts with low activity coefficients (such as K_2SO_4), on these grounds, would be predicted not to inhibit LPL activity and assays containing up to 0.6 M K_2SO_4 (close to saturation for this salt) in the presence of apoVLDL were not significantly inhibited.

pH dependence of LPL activity

"Previous results (14) had indicated that apoVLDL induced a significant alkaline shift in the alkaline limb of the plot of pH dependence of the V_{max} of LPL activity but with $pK_a = 7.0$ for both activated and nonactivated systems." As shown in **Fig. 4** an

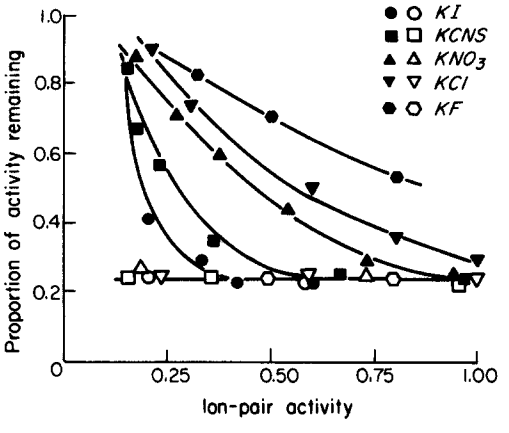

Fig. 3. Inhibition of LPL activity by potassium salts. Maximal reaction velocities in the presence and absence of apoVLDL were calculated as described in the legend for Fig. 2. Open symbols, −apoVLDL; closed symbols, +apoVLDL.

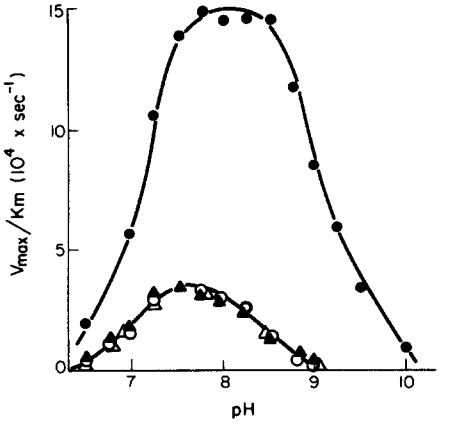

Fig. 4. pH dependence of LPL activity with triolein substrate in the presence and absence of apoVLDL (5 μg/μmole of triglyceride) and 1 M NaCl. Reaction was under the following conditions: ○—○, 0.1 M NaCl medium, in the absence of apoVLDL; △—△, 1.0 M NaCl medium, in the absence of apoVLDL; ●—●, 0.1 M NaCl medium, in the presence of apoVLDL; ▲—▲, 1.0 M NaCl medium, in the presence of apoVLDL. Assays were for 10 min at 37°C.

increase of pK_b of about 0.8 pH units that was induced by apoVLDL was reversed in the presence of 1 M NaCl to the value characteristic of the nonactivated system in these experiments. The existence of this specific effect has been previously reported (14). A plot of the pH dependence of lipase activity in the absence of apoVLDL showed that 1 M NaCl was without effect. The plot of pH vs. V_{max}/K_m represents the ionization of the free enzyme species (26) and the pK values are independent of the number of intermediate steps of product formation. Since the differences in pK values (± apoVLDL) derived from the plot of V_{max}/K_m were similar to those previously reported for V_{max} vs. pH (14), the effect of apoVLDL on LPL activity appears to be mediated at the level of free enzyme, i.e. by formation of an enzyme–cofactor complex prior to reaction with the substrate.

DISCUSSION

Basal (nonactivated) hydrolytic activity by LPL isolated from postheparin plasma is a function of medium hydrogen ion concentration, substrate concentration, and the nature of the substrate dispersion (14). This activity has been shown to be a function of this enzyme, rather than of the second (hepatic) postheparin lipase, by the following criteria: basal activity of purified enzyme was greater at pH 7.0 than at alkaline pH (Table 1), basal activity was not activated by 1 M NaCl (Fig. 2), purified LPL showed negligible phospholipase activity (Table 3), and it was

TABLE 6. Inhibition of lipoprotein lipase by monovalent ions

Anion	Concentration for 50% inhibition	
	Ion--pair activity	Molarity
I^-	0.19	0.27
CNS^-	0.22	0.32
NO_3^-	0.34	0.69
Cl^-	0.47	0.72
F^-	0.62	0.98

The concentration of each anion (as the potassium salt) required to inhibit activation of LPL by 50% was determined with triolein substrate complexed with 5 μg/μmole of apoVLDL protein, at pH 7.0 and 37°C.

not inhibited by 10^{-6}M diethylnitrophenylphosphate (2). The opposite properties have been identified for hepatic postheparin lipase (2, 13, 27, 28). Basal lipolysis is minimal at the pH optimum of activation and conditions can be chosen for which the rate of basal lipolysis is essentially undetectable (14). The purposes of the present study were quite the opposite, and conditions were chosen to permit accurate determination of both basal and activated rates. Under the same experimental conditions the degree of activation of purified LPL species from postheparin plasma, milk, and adipose tissue was not significantly different (7).

The studies reported in Table 2, carried out with a variety of natural and synthetic lipoprotein substrates, suggested that there was a relationship between the extent of activation of the LPL reaction by VLDL protein (apoVLDL), under defined conditions, and the extent of inhibition by 1 M NaCl under the same conditions. More detailed analysis in subsequent experiments has indicated this to be the case for a number of other salts.

On the other hand, reaction of the enzyme in the absence of added apoVLDL was in no case salt-dependent. Analysis of inhibition of LPL activation by NaCl showed a median effect at a mean ion-pair activity of 0.47, equivalent to about a 0.72 M salt solution (**Table 6**). When other salts tested were arranged in order of their ability to inhibit the LPL reaction, the series obtained showed striking similarities to other Hofmeister or lyotropic series for the effects of salts on protein–protein interactions expressed in terms of enzyme activities, binding affinities, electrophoretic mobilities, or mean molecular weights. Such effects have been shown to be dependent not upon molar salt concentration, nor upon "ionic strength" (which is an invalid measure of effective ion concentration for most salts at molar concentrations of greater than 0.05) (20), but upon mean ion-pair activities. The existence of such a Hofmeister series for LPL activation suggested that the effects of salts on the activation process were mediated through protein–protein interactions. Further evidence was provided by the absence of inhibition of activation by salts with low activity coefficients (such as K_2SO_4), even at high concentrations.

Inhibition of LPL activity by salts was fully reversible (Fig. 1). Inhibition was without effect on $K_{m(app)}$ for triglyceride, i.e. it was noncompetitive with apoVLDL (Table 2). Inhibition by salt was not associated with the dissociation of lipase from its substrate (Table 4) or of lipoprotein protein from the activated complex. As discussed above, the effect of apoVLDL was probably expressed at the level of the free enzyme (Fig. 4). It therefore appears unlikely that the effect of salt is expressed through a classical competitive interaction with either the cofactor or the enzyme protein species, and the back-reaction is probably not significant. It was previously shown that the reaction of apoVLDL with the LPL system was associated with a displacement to the alkaline side of the basic ionization constant (pK_b) derived from the plot of log V_{max} vs. pH, i.e. the approximation of positive charge to the enzyme active site residues involved in the rate-limiting step of catalysis. Inhibition of the catalytic rate of LPL by salts involved not only the effect on V_{max} but also the effect on pH dependence (Fig. 4).

Protein–protein association inhibited by salts can involve either like subunits (as in dimerization reactions) (10) or unlike subunits (as in formation of antibody–antigen complexes) (29). The present reaction appeared to involve unlike subunits, and it was of interest to consider whether the anion or the cation component of the salt was rate-determining in reaction with the enzyme or cofactor protein component. (There is no requirement to specify binding sites for both ions because one cannot be added to a neutral solution without the other). In considering the first factor, inhibition curves were determined for a number of salt ion-pairs. Comparison of Figs. 2 and 3 clearly indicates that, while different cations with the same anion had almost identical inhibition plots, different anions had significantly different effects. Concerning the second factor, because the effect of added salts was to *reduce* pK_b to the value characteristic of the nonactivated enzyme reaction, this should involve addition of negative charge to permit ionization of the enzyme active site at a lower hydrogen ion concentration. Consequently, if inhibition of LPL by salts is anion-specific, and involves anion binding, binding is likely to be to the cofactor protein, not to the enzyme protein moiety.

In these experiments the reaction of whole

apoVLDL protein with LPL, triglyceride, and salts has been considered. Although a single polypeptide component of apoVLDL is the effective cofactor for activation with this LPL system (3–7), use of the entire protein moiety has appeared preferable in view of the several modifying effects of other apolipoproteins that have been reported (7, 8). Additionally, it has been shown in the present study that the proportions of proteins complexed with triglyceride substrate in the presence of high salt concentrations are essentially similar to those present at low (non-inhibiting) ion concentrations. Consequently, the assumption that the modifying effects of non-cofactor lipoprotein proteins are unchanged by salts appears reasonable. Although there is presently no evidence that the β-protein moiety of apoVLDL plays a role in the LPL reaction, data reported here confirm that the techniques used in this investigation also bind this protein species to a triglyceride–lecithin complex.

This study has been limited to uni-univalent salts, except for K_2SO_4. While further information could be gained with a greater range of atomic volumes (30), mean ion-pair activity for inhibition of this reaction is quite high and the activity coefficients for polyvalent ions are rather low in many cases. In addition, many salts either induce irreversible inactivation of the LPL system (such as $HgCl_2$) or are incompatible with enzyme assay techniques (such as CH_3COO^-). However, the series utilized in this research includes salts with major (KI, KCSN, LiCl) and minor (KF) effects on protein hydration and a considerable range of activity coefficients.

The evidence obtained in this study suggests that the effects of inorganic salts on the LPL reaction are mediated at the level of the enzyme–apoprotein complex, are primarily a function of the salt anion, and probably involve reaction with a positively charged cofactor protein site. The data are compatible with those obtained in several other studies of salt–protein interaction and the Hofmeister series for LPL inhibition by salts is similar to those reported for these systems. The study suggests that the characteristic features of the LPL reaction, its activation by lipoprotein protein and its inhibition by inorganic salts, may be linked functions.

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