Differential characteristics of purified hepatic triglyceride lipase and lipoprotein lipase from human postheparin plasma

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Abstract Evidence is presented that hepatic triglyceride lipase (H-TGL) and lipoprotein lipase (LPL), purified from human postheparin plasma, can each hydrolyze both glyceryl trioleate and palmitoyl-CoA. The average ratio of glyceryl trioleate/palmitoyl-CoA hydrolase activities, obtained with enzyme preparations from 15 human postheparin plasma samples was 1.30 (1.18–1.52) for H-TGL and 8.75 (7.45–10.25) for LPL. Albumin was identified as the serum cofactor required for the hydrolysis of palmitoyl-CoA by H-TGL. It protected this enzyme from inactivation by this substrate. In contrast, palmitoyl-CoA activated and protected LPL from denaturation by dilution and incubation at 25°C. The effects of other detergents were investigated on glyceryl trioleate hydrolase activities of both enzymes. Sodium dodecyl sulfate (0.4 mM) and Tris-oleate (0.4 mM), which also effectively activated and protected LPL against inactivation, had only moderate protective effect on H-TGL. Sodium dodecyl sulfate at a higher concentration (1 mM) produced little or no inhibition of LPL, while completely inactivating H-TGL. Conversely, sodium taurodeoxycholate (0.4 mM) protected and activated H-TGL, but had only moderate protective effect on LPL. Triton X-100 (0.1–0.8 mM) and egg lysolecithin (0.05–2 mM) also protected H-TGL, but not LPL. The very dissimilar effects of detergents on preparations on H-TGL and LPL may form the basis for the direct assay of each enzyme in the presence of the other.

Supplementary key words glyceryl trioleate hydrolase · palmitoyl-CoA hydrolase · detergents

Hepatic triglyceride lipase (H-TGL) and lipoprotein lipase (LPL) (glycerol ester hydrolase EC 3.1.1.3) have been isolated from postheparin plasma of human subjects (1–6) and other mammals (7–10). Although active against similar substrates, they differ in their tissues of origin and in many of their biochemical characteristics. Among the latter is the requirement for an apolipoprotein cofactor in the hydrolysis of triglycerides by LPL (11, 12), but not by H-TGL (2, 4, 8). This cofactor, apoC-II[1], has been isolated from the very low density lipoproteins (13–15) and is also found in the high density lipoproteins (16, 17) of human plasma. Other apolipoproteins have also been found to increase the activity of lipolytic and lipid-transfer enzymes. Thus, another apolipoprotein from the very low density lipoproteins, apoC-I, has been reported to activate a postheparin lipoprotein lipase (18, 19), different from LPL. Also, apoC-I (20), as well as apoA-I (21, 22) and apoA-III[2] (23, 24), apolipoproteins isolated from the high density lipoproteins, have been found to stimulate the reaction catalyzed by lecithin:cholesterol acyltransferase.

Recently, postheparin plasma samples of rat and man have been found to have thioesterase activity that uses palmitoyl-CoA as substrate. This activity has been attributed primarily to H-TGL (29, 30). The report that serum contained a cofactor required for palmitoyl-CoA hydrolysis suggested the possible role of yet another apolipoprotein in lipolytic reactions. This was of added interest since a specific cofactor for H-TGL has not yet been described.

In the present work, the glyceryl trioleate and palmitoyl-CoA hydrolase activities of H-TGL and LPL, purified from human postheparin plasma, were compared. The serum factor required for palmitoyl-CoA hydrolysis by H-TGL was identified, and its mode of action was determined. Since palmitoyl-CoA has been reported to have detergent-like properties (31, 32) and its effects on H-TGL and LPL were remarkably different, the actions of other deter-

Abbreviations: H-TGL, hepatic triglyceride lipase; LPL, lipoprotein lipase; apo-, apolipoprotein; CoA, coenzyme A; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); FFA, free fatty acids; Tris, tris(hydroxymethyl)amino methane; DOC, sodium deoxycholate; TDOC, sodium taurodeoxycholate; SDS, sodium dodecyl sulfate.

[1] The nomenclature of the apolipoproteins suggested by Alaupovic (25) was used in this paper. The alternative classification based on the carboxy-terminal amino acids (26) is given in parenthesis: apo-CI (apo-Lp-ser); Apo-CII (apo-Lp-glu); apo-CIII (apo-Lp-ala); apo-AI (apo-Lp-glnI); apo-AII (apo-Lp-glnII).

[2] Also called "thin line" polypeptide or apolipoprotein D (27).

gents on the two enzymes were also compared.

EXPERIMENTAL PROCEDURES

Materials

Heparin–Sepharose was prepared by covalently linking crude heparin derived from intestinal mucosa (Wilson Laboratories, Chicago, IL) to Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the method of Iverius (33). Concanavalin A–Sepharose gel, Sephadex G-100, chymotrypsinogen A, and ribonuclease were also from Pharmacia Fine Chemicals. Methyl-α-D-glucopyranoside and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) were from Calbiochem, San Diego, CA; Coenzyme A derivatives of palmitic, oleic, and linoleic acids were obtained from Avanti Biochemicals, Inc., Birmingham, AL; palmitic and oleic acids, sodium deoxycholate and taurodeoxycholate, and Triton X-100 were from Sigma Chemical Co., St. Louis, MO. Glyceryl trioleate (unlabeled) was from Nu-Chek Prep, Inc., Elysian, MN; glyceryl tri-[1-^{14}C]oleate was obtained from Dhom, North Hollywood, CA; sodium decyl and dodecyl sulfates from Eastman (Rochester, NY), egg lysolecithin from General Biochemicals (Chagrin Falls, OH), rabbit gamma globulin (Fraction II) from Pentex (Kankakee, IL) and bacitracin from Swartz-Mann (Orangeburg, NY). Fatty acid-free albumins were prepared by treating bovine serum Fraction V, crystallized bovine serum albumin (Reheis Chemical Co., Chicago, IL), or normal human albumin (USP, Cutter Labs., Berkeley, CA) with charcoal, according to the procedure of Chen (34). Palmitic acid was added to some preparations of the fatty acid-free human albumin using the method of Spector and Hoak (35).

Enzyme preparations

Blood was collected from human normal subjects 15 min after injection of 60 U of sodium heparin derived from intestinal mucosa (Riker Labs., Inc.) per kg body weight. The plasma was diluted immediately with an equal volume of 0.005 M sodium veronal–0.45 M NaCl buffer, pH 7.0, to obtain a final NaCl concentration of approximately 0.3 M. (A NaCl concentration of about 0.3 M yielded maximal adsorption of H-TGL and LPL from postheparin plasma to the Heparin–Sepharose gel).[3] The diluted plasma samples were then applied to heparin–Sepharose columns (1 ml of gel was adequate for 6–8 ml of diluted plasma) and the enzymes were eluted using stepwise increases of NaCl concentration in the veronal buffer, as described previously (36, 37). H-TGL and LPL, which had been purified further by chromatography on concanavalin A–Sepharose gel, were used in some experiments. The enzymes, as eluted from the heparin–Sepharose column (H-TGL in 0.005 M sodium veronal–0.75 M NaCl, and LPL in 0.005 M sodium veronal–1.5 M NaCl, both buffers at pH 7.0) were directly applied to columns of concanavalin A–Sepharose gel and eluted as previously described for H-TGL (6), except that the buffers used in the washing and elution steps also contained 10 mM $CaCl_2$ and 1 mM $MgCl_2$[4]. Chromatography on heparin–Sepharose gel resulted in more than 2,000-fold purification of both enzymes (37); the subsequent chromatography on concanavalin A resulted in a 2- and 4-fold further purification of H-TGL and LPL, respectively[4]. The enzymes thus prepared could be stored for several months at −80°C with only slight loss of activity. No qualitative differences in the results were observed when using either freshly prepared or frozen enzymes. Unless otherwise stated, frozen enzymes at the stage of purification obtained after heparin–Sepharose chromatography were used in these experiments.

Apolipoproteins

VLDL was obtained from the plasma of patients with Type IV hyperlipoproteinemia, as described previously (13). The lyophilyzed sample was delipidated by extraction once with heptane, three times with diethyl ether–absolute ethanol 1:1, once with benzene, and once with diethyl ether. (All solvents were at 0°C or lower.) The apoC lipoproteins were then obtained by a modification of the method previously described for the isolation of apoC lipoproteins from rat serum (38). The delipidated sample was dissolved in a buffer containing 0.05 M Tris-HCl–6 M urea–0.1 M sodium decyl sulfate. Gel filtrations were carried out on a column of Sephadex G-100 with a buffer containing 0.05 M Tris-HCl–6 M urea–0.001 M decyl sulfate and 0.02% sodium azide. A linear gradient using equal volumes of 0.005 M Tris-HCl–6 M urea and 0.1 M Tris-HCl–6 M urea–0.04 M NaCl buffers was employed for the separation of the apolipoproteins by chromatography on DEAE cellulose. The pH of all buffers was 8.2.

ApoA-I and apoA-II were prepared from the high

[3] Boberg, J., J. Augustin, M. L. Baginsky, P. Tejada and W. V. Brown. p 544–547. This journal.

[4] Augustin, J., H. Freeze, J. Boberg, and W. V. Brown. Unpublished data.

density lipoproteins (d 1.063–1.21) of plasma from normal fasting donors, as described previously (39).

Protein concentrations were determined by the method of Lowry et al. (40), using bovine serum albumin as standard.

Determination of triglyceride hydrolase activity

Hydrolysis of glyceryl trioleate by H-TGL was measured at pH 8.8 in 0.2 M Tris-HCl–0.75 M NaCl, without added activator lipoprotein. LPL was assayed at pH 8.2 in 0.2 M Tris-HCl–0.08 M NaCl and 20 μg of apoC-II were added per ml of assay mixture. The assay mixtures (0.30–0.46 ml) for both enzymes contained 10 mg/ml of fatty acid-free albumin and 2.3 μmol/ml of glyceryl tri[1-^{14}C]oleate (sp act 0.109 μCi/μmol), emulsified by sonication with 5% gum arabic in 0.2 M Tris-HCl at the appropriate pH. The final concentration of gum arabic was 7.5 mg/ml of assay mixture. The reaction was started by addition of 10–50 μl of enzyme solution, which in no case hydrolyzed more than 10% of the substrate in 30 min at 28°C. The free fatty acids produced were extracted and determined by the partition system described by Pittman, Khoo, and Steinberg (41).

Palmitoyl-CoA hydrolase activity

Palmitoyl-CoA hydrolase activity was measured in terms of the chromophore formed between reduced CoA and DTNB. This was determined by the absorbance at 412 nm measured with a Gilford 2400 spectrophotometer at 30°C. The concentration of the chromophore was calculated using the molar extinction coefficient of 13.6×10^3. The routine assay mixtures contained 100 μmol of Tris-HCl (pH 8.5), 150 μmol of NaCl, 0.5 μmol of $CaCl_2$, 0.75 mg of fatty acid-free albumin, 0.15 μmol of palmitoyl-CoA, 0.2 μmol of DTNB, and 20–100 μl of enzyme solution, in a total volume of 1.0 ml. The baseline absorbance at 412 nm of mixtures containing all components except enzyme was recorded for 6–8 min. Hydrolysis of palmitoyl-CoA was determined by the rate of change in absorbance at 412 nm during the first 2–4 min after addition of the enzyme. Fresh solutions of DTNB and palmitoyl-CoA were prepared for each experiment. Acyl-CoA concentrations in these solutions were determined by measuring absorbance of 259 nm after dilution in 0.1 M Tris-HCl (pH 7.0). A molar extinction coefficient of 15.4×10^3 was used.

The term "pre-incubation" refers to specified periods during which the enzymes were exposed to a given set of conditions prior to their assay with either glyceryl trioleate or palmitoyl-CoA as substrate.

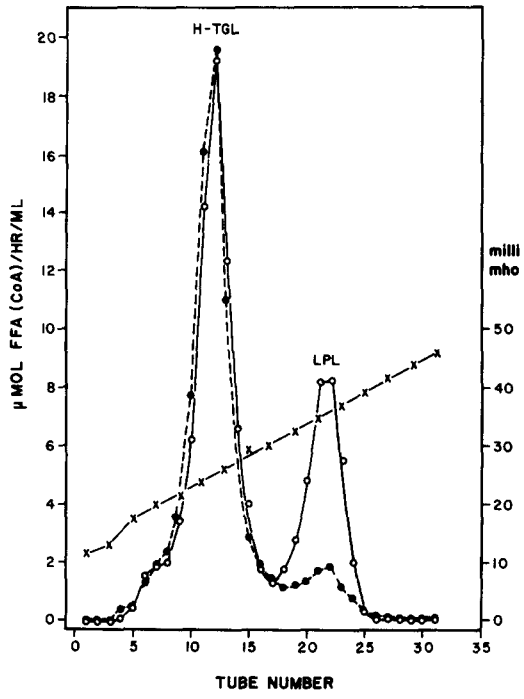


Fig. 1. Column chromatography of human postheparin plasma on heparin–Sepharose gel. Twenty ml of postheparin plasma was applied to a heparin–Sepharose column (1.6 × 2.6 cm) as described in the Methods section. The column was then washed with 100 ml of 0.3 M NaCl in 0.005 M sodium veronal buffer, pH 7.0, and the enzymes were eluted with 150 ml of this veronal buffer containing a concentration gradient from 0.3 M to 2.0 M NaCl. Four ml fractions were collected (50 ml/hr). Glyceryl trioleate hydrolase activity (○ —— ○) was assayed as indicated in the text, using 60 μl of preheparin serum per ml of assay mixture as the source of activator apolipoprotein. Palmitoyl-CoA hydrolase activity (● —— ●) was determined as indicated in the Methods section. Conductivities (x —— x) were measured on the samples at 0°C.

RESULTS

Glyceryl triolate and palmitoyl-CoA hydrolase activities of H-TGL and LPL

The elution pattern of the glyceryl trioleate hydrolase activities obtained by chromatography of human postheparin plasma on the heparin–Sepharose column coincided with that of the palmitoyl-CoA hydrolase activities (**Fig. 1**). As described for rat plasma (30), the fractions containing the H-TGL also had most of the palmitoyl-CoA hydrolase activity. In the experiment shown in Fig. 1, the glyceryl trioleate hydrolase activity of H-TGL was lower than maximal since all fractions were assayed under the conditions defined as optimal for LPL (see Methods section). Also, preheparin serum, used here as the source of activator apolipoprotein for LPL, was frequently found to inhibit the hydrolysis of glyceryl trioleate by H-TGL.

The cochromatography of the palmitoyl-CoA and

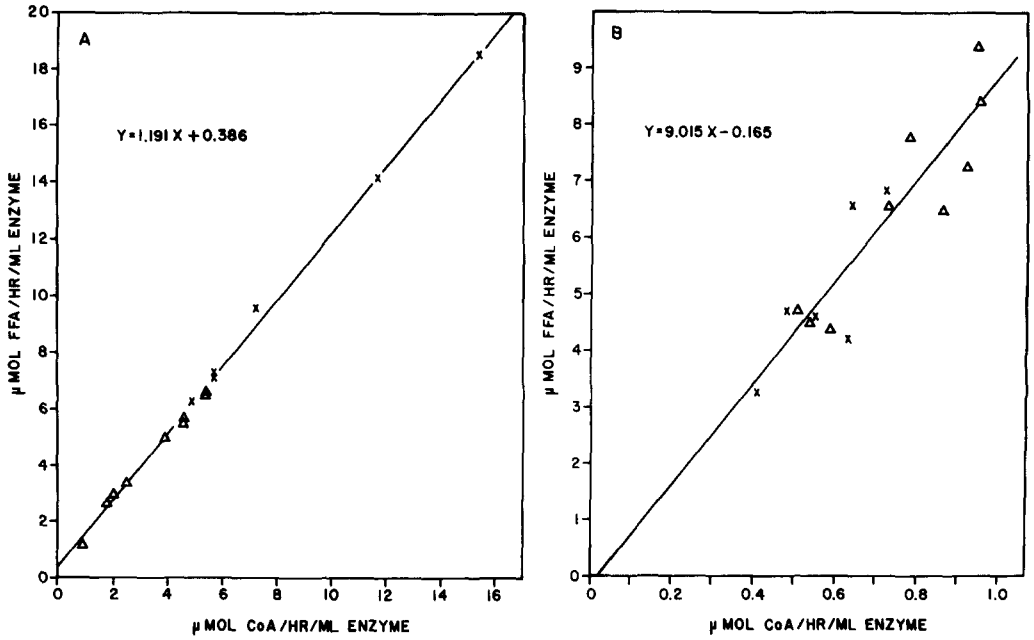


Fig. 2. Correlation between the glyceryl trioleate and palmitoyl-CoA hydrolase activities of H-TGL and LPL. Glyceryl trioleate hydrolase activities (ordinate) and palmitoyl-CoA hydrolase activities (abscissa) were determined in preparations of H-TGL (Fig. 2A) and LPL (Fig. 2B) purified from the postheparin plasma of 15 normal fasting subjects (6 males, x, and 9 females, △). For the data presented in Fig. 2A, glyceryl trioleate hydrolase was measured under conditions optimal for H-TGL, while in Figure 2B this activity was measured under conditions optimal for LPL (see Methods section).

glyceryl trioleate hydrolase activities suggested that each enzyme (H-TGL and LPL) is capable of hydrolysing both esters, which is also consistent with studies in the rat (30). This hypothesis was further tested by measuring the rates of hydrolysis of both substrates by H-TGL and LPL isolated from the plasma of fifteen different individuals. The two enzymes were purified from 3 ml of these postheparin plasma samples using 1 ml heparin–Sepharose columns, as previously described (36). A strong correlation was observed (**Fig. 2A**) between the levels of glyceryl trioleate and palmitoyl-CoA hydrolase activities in the purified H-TGL preparations ($r = 0.99$, $P < 0.01$). The ratio of glyceryl trioleate/palmitoyl-CoA hydrolase activities was 1.30 (1.18–1.52). Although this ratio was much higher for LPL (8.75, range 7.45–10.25), the correlation was again quite significant ($r = 0.91, P < 0.01$) (Fig. 2B). ApoC-II, added at concentrations yielding maximum activation of glyceryl trioleate hydrolysis by LPL, produced only a 5–10% increase in the rate of hydrolysis of palmitoyl-CoA by this enzyme. Therefore, its relatively low thioesterase activity was not due to the lack of this activator apolipoprotein. There was no significant correlation ($r = 0.297$) between the hydrolysis of the two substrates by purified LPL when glyceryl trioleate hydrolysis was measured under the high salt condition that suppresses LPL while optimizing H-TGL. Thus palmitoyl-CoA hydrolase activity of LPL could not be explained by contamination with H-TGL. When the postheparin plasma samples (rather than the purified enzymes) were assayed for glyceryl trioleate hydrolase activity, using the assay conditions found to be optimum for H-TGL (see Methods section), a strong correlation between glyceryl trioleate and palmitoyl-CoA hydrolase activities was again observed ($r = 0.96$, $P < 0.01$). However, no significant correlation ($r = 0.260$) between these activities was obtained when glyceryl trioleate hydrolysis was determined using the low salt plus apoC-II medium that measures the hydrolysis of glyceryl trioleate by both H-TGL (suboptimally) and LPL.

Serum cofactor(s) required for palmitoyl-CoA hydrolyase activity

In the absence of human serum or albumin, the palmitoyl-CoA hydrolase activity of H-TGL gave low rates, which decreased progressively with incubation time. Addition of 20 μl of human serum to the assay mixture was optimal, producing a threefold increase in the initial reaction rate and a more linear time course (**Fig. 3A**). Slightly higher values were achieved when 0.75 mg of fatty acid-free albumin was added instead of this amount of serum. Bovine serum al-

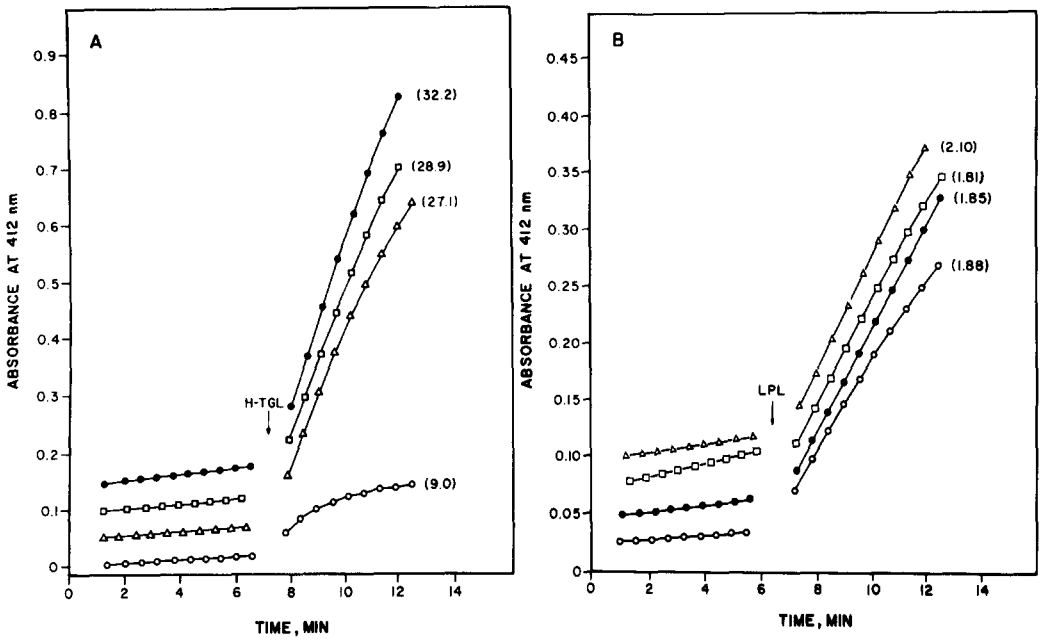


Fig. 3. The effect of human serum and bovine serum albumin on palmitoyl-CoA hydrolase activity of H-TGL and LPL. Purified H-TGL (4.8 μg protein) was used in the experiments of Fig. 3A and purified LPL (13.2 μg protein) in the experiments of Fig. 3B. Curves present: ○ —— ○, no addition; △ —— △, 10 μl of human serum added; □ —— □, 20 μl of human serum added and ● —— ●, 0.75 mg of fatty acid-free bovine serum albumin added. Values in parenthesis give the reaction rates in μmol CoA liberated/hr per ml of enzyme solution, calculated from the differences in absorbance at the initial, linear portion of the curves.

bumin (crystallized or Fraction V) or human serum albumin were equally effective. H-TGL that had been further purified by chromatography on concanavalin A–Sepharose gel also required serum or albumin for a linear rate of hydrolysis of palmitoyl-CoA. However, in the protein-free assay medium (control), initial rates were higher than those observed with the enzyme purified only by heparin–Sepharose chromatography. This was probably due to the effect of $CaCl_2$ contained in the buffer used for elution of the enzyme from the concanavalin A gel, since addition of $CaCl_2$ (5×10^{-4}M) to an assay medium (without serum or albumin) resulted in approximately 60% higher initial activity.

Addition of protein made little difference in the initial rates of palmitoyl-CoA hydrolysis by LPL. However, longer periods of linearity were observed in the presence of either serum or albumin (Fig. 3B). After further purification of LPL by concanavalin A chromatography, the initial rates were about 20% higher in the control than in assay mixtures containing serum or albumin. This result was also attributable to the $CaCl_2$ in the enzyme preparation, but apparently through a different mechanism. Thus, H-TGL was not stimulated by Ca^{2+} when assayed in a medium containing albumin, and addition of EDTA or EGTA (2 mM) to this assay medium reduced the reaction rate by less than 5%. In contrast, calcium seems to be an activator for LPL (also shown by others) (42). In assays measuring palmitoyl-CoA hydrolysis by this enzyme (as obtained from the heparin–Sepharose column), 2 mM EDTA produced 70–100% inhibition in a medium without albumin and about 50% inhibition in its presence.

The findings that fatty acid-free albumin was more effective than serum in activating H-TGL, and that higher hydrolytic rates were obtained in the protein-free assay medium when $CaCl_2$ was added, suggested that these stimulatory effects were due mainly to the removal of the released palmitic acid. This was consistent with the much less effective activation observed when albumin containing added palmitic acid was used instead of the fatty acid-free preparation. In an experiment similar to that shown in Fig. 3A, addition of 0.75 mg of fatty acid-free human albumin increased the rate of reaction 4.4-fold over control values. The same quantity of albumin containing 10 nmol of added palmitic acid gave a 2.9-fold activation, while that containing 20 nmol produced less than 1.7-fold increase in reaction rate. As previously noted, albumin was not required for palmitoyl-CoA hydrolysis by LPL. However, the condition with 0.75 mg albumin containing 10 nmol of palmitic acid gave 10–15% higher values than assay mixtures

TABLE 1. Effect of proteins on hydrolysis of palmitoyl-CoA by H-TGL

Protein Added	H-TGL after Hep.–Seph. Chromatography		H-TGL after Con. A Chromatography	
	Activities[a]	%	*Activities*[a]	%
Human serum albumin	14.3[b]	(100)	13.8[c]	(100)
None	5.5	38.5	3.4	24.8
Apo-AI	11.7	81.8	12.1	87.9
Apo-AII	10.8	75.5	7.7	56.1
Apo-CI			7.9	57.7
Apo-CII	6.8	47.6	7.9	57.7
APO-CIII[d]	7.5	52.4	5.5	40.0
Apo-CIII (0.5 mg)	8.2	57.3	10.6	76.9
Rabbit gamma globulin	5.5	38.5	6.4	46.5
Chymotrypsinogen A			7.3	52.8
Ribonuclease			4.6	33.6
Bacitracin			3.5	25.6
Bacitracin (0.5 mg)			7.3	52.8

[a] μmol/hr/ml enzyme solution.
[b] Average, n = 7, range 13.2–15.0.
[c] Average, n = 9, range 12.1–15.0.
[d] The form containing one sialic acid moiety (13) was used in these experiments. Protein values obtained with the method of Lowry et al. (40) were multiplied by 0.85 to correct for true protein.

Assays of palmitoyl-CoA hydrolase activity were carried out as described in the Methods section, except that the assay media contained (per ml): 100 μmol of Tris-HCl (pH 8.0), 7.5 μmol of ammonium bicarbonate, 2 μmol of EDTA, 0.081 μmol of palmitoyl-CoA, 0.2 μmol of DTNB, and 7.5 nmol (unless otherwise indicated) of the various proteins. The H-TGL protein added to each assay was 3.2 μg as purified by heparin–Sepharose or 2.1 μg for the preparation after further chromatography on concanavalin A. The molecular weight used for the several proteins were: human albumin, 69,000; apo AI, 28,000; apo AII, 17,000; apo-CI, 7,000; apo-CII, 10,000; apo-CIII, 9,000; rabbit gamma globulin, 160,000; chymotrypsinogen A, 25,000; ribonuclease, 13,000 and bacitracin, 1,450.

In each set of four samples in the Gilford 2400 spectrophotometer a control mixture containing albumin was monitored simultaneously with the protein tested. The data given for albumin are the averages of all the values obtained with each enzyme preparation. The activity given for each protein is the experimentally determined value (measured during the first minute of assay after enzyme addition), normalized to the average value for albumin.

without albumin, with albumin alone, or with albumin plus 20 nmol of palmitic acid.

Addition of albumin (0.75 mg), 10 min after the enzyme, to the assay medium measuring hydrolysis of palmitoyl-CoA by H-TGL, did not restore this enzyme activity. As will be shown in later sections, albumin is required by both H-TGL and LPL for continued hydrolysis of glyceryl trioleate (Fig. 5). It is believed that in these reactions albumin acts principally as a fatty acid acceptor. However, in the hydrolysis of palmitoyl-CoA by H-TGL, albumin, as well as serum and Ca^{2+}, has the additional effect of protecting the enzyme from its inactivation by palmitoyl-CoA (see Table 2). This is in agreement with the findings that the ratio of palmitoyl-CoA (or oleyl- or stearoyl-CoA, which are also substrates for H-TGL and LPL) to albumin was more critical than the absolute concentrations of these acyl derivatives in obtaining maximal rates of hydrolysis by the two enzymes. A ratio of about 0.5 mg (7.25 nmol) of fatty acid-free albumin to 100 nmol of palmitoyl-CoA was found to be optimum.

The effect of nine other proteins on the hydrolysis rates of palmitoyl-CoA by H-TGL was compared on a molar basis (7.25 nmoles/assay) and on a weight basis (0.5 mg protein/assay) to that observed with albumin. Only one preparation of each protein was tested. In these experiments H-TGL preparations obtained after heparin–Sepharose column chromatography and after further purification on concanavalin A were used. A summary of a typical experiment is given in **Table 1.** None of the proteins tested was as effective as albumin. Only apoC-III and bacitracin gave higher initial values when 0.5 mg of protein, rather than 7.25 nmol, was added. The other proteins gave the same or lower values under this condition.

Pre-incubation of H-TGL and LPL with oleic acid

The potassium salts of oleic, linoleic, and linolenic acids have been used by Fielding et al. (1. 9, 43) to protect LPL from inactivation during its isolation from postheparin plasma. Since no studies have been reported on the effect of fatty acids on H-TGL, a comparative study of the effect of oleic acid on the two purified enzymes was performed. The Heparin–Sepharose column eluates containing the H-TGL or LPL were diluted 5- and 2.5-fold, respectively, (to attain a final concentration of 50 μg of enzyme protein/ml) in 0.1 M Tris-HCl–1 mM EDTA buffer, pH 8.5, containing various amounts of oleic acid. After a 10-min pre-incubation at 28°C, the activities of both enzymes were determined using glyceryl trioleate (**Fig. 4A**) and palmitoyl-CoA (**Fig. 4B**) as substrates. The results are expressed as percent of activity relative to that of the undiluted enzyme (no oleic acid), under otherwise identical conditions. Dilution of LPL with buffers without oleic acid resulted in more than 80% loss of activity (Fig. 4A and 4B). Addition of oleic acid completely prevented this inactivation and, in fact, increased the activity toward both glyceryl trioleate (Fig. 4A) and palmitoyl-CoA (Fig. 4B) beyond that seen with the undiluted preparation. The activity of H-TGL was less affected by dilution than that of LPL. Low levels (0.1 mM) of oleic acid were also protective for H-TGL, but the higher concentrations of oleic acid, which were optimal for protection of the LPL, inhibited the activity of H-TGL. The greater inactivating effect of oleic acid observed for H-TGL when assayed with palmitoyl-CoA (as compared with glyceryl trioleate) is due, at least in part, to inhibition of this reaction by oleic acid,

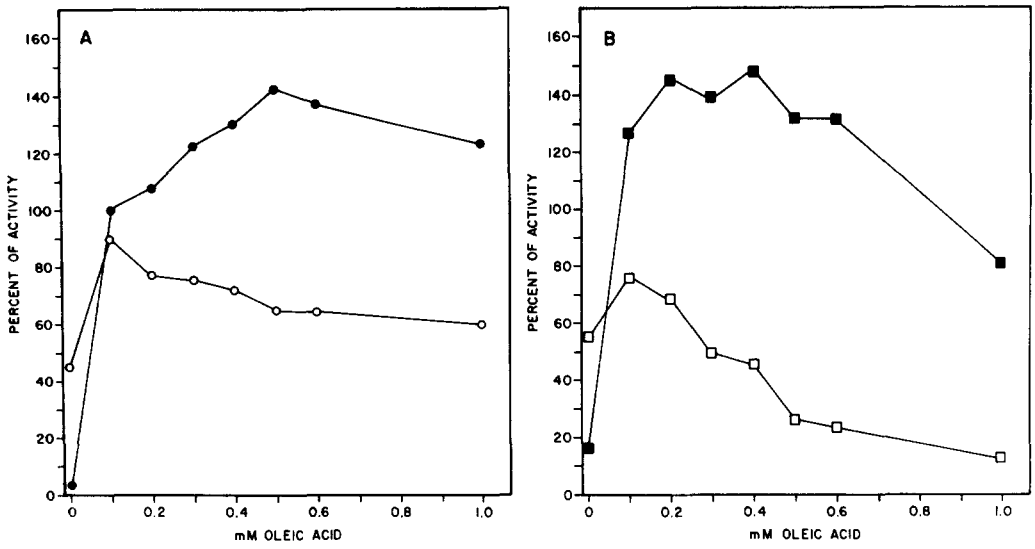


Fig. 4. Effects of pre-incubation of H-TGL and LPL with oleic acid. Clear solutions of oleic acid in 0.1 M Tris-HCl–1 mM EDTA buffer (pH 8.5) were obtained by sonication. After pre-incubation the samples were assayed with glyceryl trioleate (Fig. 4A) or palmitoyl-CoA (Fig. 4B) substrates. Final concentrations of enzyme and oleic acid in the assay media were 1/9 of those in the pre-incubation mixtures. H-TGL ○ —— ○, □ —— □; LPL ● —— ●, ■ —— ■.

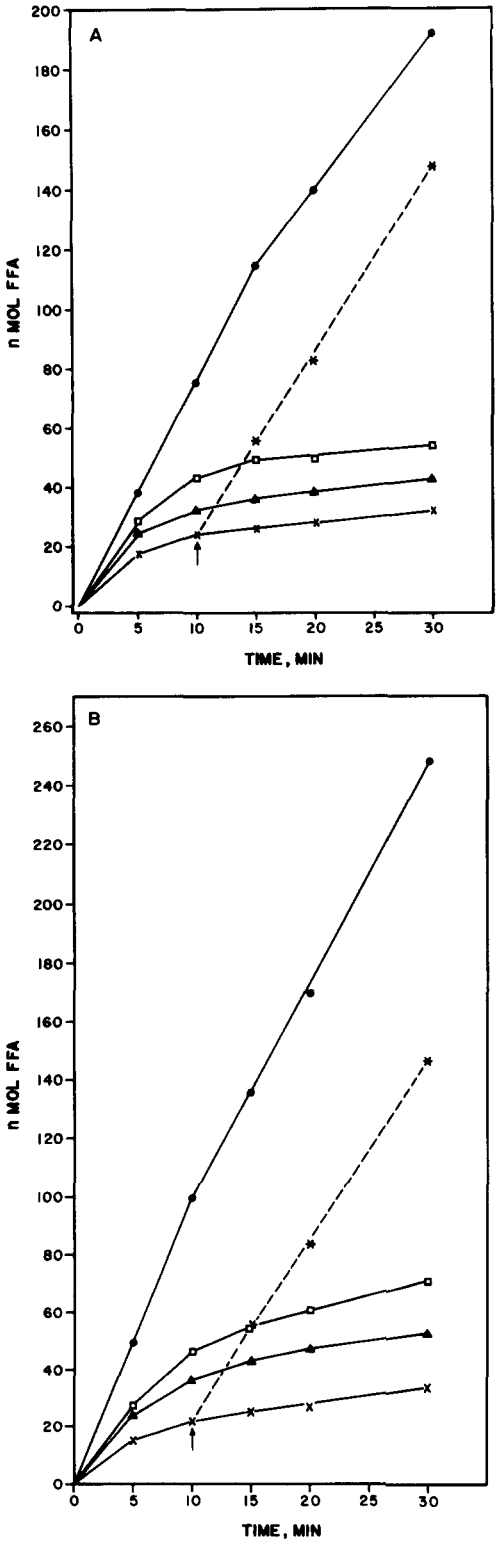
rather than to enzyme inactivation. The assay mixture for the measurement of palmitoyl-CoA hydrolysis contains 1/6 the amount of albumin used in the assay medium for glyceryl trioleate hydrolysis. Reduction of the degree of inhibition of H-TGL by oleic acid, in the palmitoyl-CoA assay, could be obtained by increasing the amount of albumin (and palmitoyl-CoA).

Mechanisms of stimulation by albumin

In view of the difference in the requirements for albumin observed with H-TGL and LPL in the palmitoyl-CoA hydrolytic reaction, it was of interest to determine whether similar effects also occurred when glyceryl trioleate was the substrate. The data of **Fig. 5** show that, for both enzymes, the rate of glyceryl trioleate hydrolysis depended upon the amount of albumin present in the assay medium. This dependence was somewhat more pronounced with H-TGL (Fig. 5A) than with LPL (Fig. 5B). The reduction of the reaction rate with time in the medium with little or no albumin was most probably due to accumulation of unbound fatty acid. Thus, hydrolysis of glyceryl trioleate by both H-TGL and LPL resumed, with rates similar to the initial rates of the control samples, upon addition of albumin 10 min after the enzyme (* – – – *, Fig. 5A and 5B). This contrasted with the previously mentioned finding that the palmitoyl-CoA hydrolase activity of H-TGL was not restored when albumin (0.75 mg) was added in an analogous experiment (data not shown). Thus, an additional role for albumin in assay systems measuring hydrolysis of palmitoyl-CoA is indicated.

Inhibitory effects of palmitoyl-CoA on several enzymes have been reported (31, 32, 44) as has the binding of this acyl derivative to proteins (31, 45, 46). The protective effect of proteins against inactivation of enzymes in the presence of palmitoyl-CoA has been explained by this mechanism. To test whether a similar inactivation of H-TGL by palmitoyl-CoA occurred, this enzyme was pre-incubated for 5 min (at 25°C) in Tris-HCl buffer containing increasing amounts of palmitoyl-CoA, with and without albumin, and then assayed using the glyceryl trioleate assay system. Analogous experiments were also conducted with LPL. The results shown in **Table 2** were obtained using enzymes purified on the day of the experiment and not frozen. The values presented for the mixtures containing palmitoyl-CoA are relative to the data obtained with the enzymes diluted in buffer without this thioester. Whereas low levels of palmitoyl-CoA (25–50 μM) protected H-TGL from inactivation during pre-incubation, inhibition occurred when the concentration of the acyl derivative was increased. No such inhibitory effect of palmitoyl-CoA was observed when the pre-incubation mixture contained albumin. In contrast to H-TGL, LPL was protected and/or activated by palmitoyl-CoA at all of the levels tested, and this effect also was much less evident in the presence of albumin. Disaggregation of LPL seems a likely mechanism for activation by palmitoyl-CoA, a compound known to have detergent properties (31, 32). Since aggregation is likely to in-

crease by freezing, a parallel experiment was done using LPL that had been kept frozen for several months. The activation of this enzyme was much more dramatic than that observed with the freshly isolated preparation (Table 2).



It has been observed repeatedly that the activity of highly purified LPL decreased rapidly on standing, even at 0–4°C. This inactivation could also be due to aggregation of the enzyme and/or its denaturation at the water–air interface (see Discussion section). The results summarized in Table 2 are consistent with the hypothesis that palmitoyl-CoA is not only a substrate for both H-TGL and LPL, but also a detergent that activates LPL and inhibits H-TGL.

Effects of other detergents

The effects of other detergents were studied to test the hypothesis that the activating (and inhibitory) effects of palmitoyl-CoA were indeed related to its detergent properties. As shown in **Fig. 6,** the enzyme preparations were diluted with Tris-HCl buffer containing the various detergents and incubated at 25°C before assay for glyceryl trioleate hydrolase activity. The concentrations of detergents in this experiment were those found to yield the highest activity of either H-TGL or LPL when assayed after a 5-min pre-incubation at 25°C. Controls using undiluted H-TGL and LPL, or these enzymes diluted in Tris-HCl without detergents, were also included. Incubation of H-TGL (Fig. 6A) with 0.5 mM taurodeoxycholate resulted in activities higher than those observed with the undiluted control. Protection from inactivation by dilution was also obtained with 0.8 mM Triton X-100 and 5 mM sodium deoxycholate and, to a much lesser degree, by 0.4 mM sodium dodecyl sulfate and 0.4 mM Tris-oleate. On the other hand, pre-incubation with 0.2 mM palmitoyl-CoA resulted in a more rapid loss of activity than that observed with buffer alone. LPL was markedly differently affected by the same detergents at the same concentrations (Fig. 6B). Activity higher than that in the undiluted control was obtained when LPL was diluted with each of the detergent-containing buffers[5], and this higher activity was maintained throughout

[5] The apparent lesser degree of activation of LPL by detergents shown in Fig. 6B, as compared with the data of Table 2, is due to the control sample chosen to normalize the values, namely, undiluted control for the data in Fig. 6B and diluted control for the data of Table 2.

Fig. 5. Requirement for albumin in the hydrolysis of glyceryl trioleate by H-TGL and LPL. Assay conditions, except that different amounts of fatty acid-free bovine serum albumin were added, were as described in the Methods section. Assay mixtures contained 2.4 μg of purified H-TGL protein (Fig. 5A) and 3.3 μg of purified LPL protein (Fig. 5B) in a final volume of 0.45 ml. The curves represent assays with: x —— x, no albumin added; ▲ —— ▲, 0.5 mg/ml albumin; □ —— □, 1.0 mg/ml albumin; ● —— ●, 5 mg/ml albumin; ★ — — — ★, 5 mg/ml albumin added after 10 minutes to the medium without albumin.

TABLE 2. Effects of pre-incubation with palmitoyl-CoA, with or without albumin, on glyceryl trioleate hydrolase activities of H-TGL and LPL

Additions during Pre-incubation		H-TGL (not frozen)		LPL (not frozen)		LPL (frozen)	
	μM P-CoA[a]	*nmol*[b]	%	*nmol*[b]	%	*nmol*[b]	%
No albumin	0	62.8	(100)	24.2	(100)	8.4	(100)
	25	72.2	115	92.2	382	144.7	1718
	50	64.6	103	97.5	404	167.8	1993
	100	54.1	86	93.0	385		
	200	54.9	87	95.0	393	236.0	2802
	300	52.3	83	96.6	400	231.0	2743
Plus albumin	0	72.8	116	36.5	151	56.1	666
	25	70.5	112	32.9	136	52.8	627
	50	72.1	115	32.9	136		
	100			34.9	144	59.2	702
	200	71.0	113	34.3	142	64.2	762
	300	70.0	112	34.9	144	72.8	864

[a] Palmitoyl-CoA.

[b] nmol of fatty acids liberated in 30 min at 28°C.

The enzymes, as obtained from the heparin–Sepharose columns were diluted (as indicated below) with 0.2 M Tris-HCl buffer, pH 8.2, containing palmitoyl-CoA to obtain the final concentrations indicated. The final concentration of fatty acid-free bovine serum albumin, where used, was 8.3 mg/ml. "Non-frozen" H-TGL and LPL were diluted 2- and 1-fold, respectively to attain the same protein concentration in the pre-incubation mixtures. In this instance this was 15 μg/ml. Frozen LPL was diluted 3-fold, giving a protein concentration of 40 μg/ml. These mixtures were pre-incubated for 5 min. at 25°C. Glyceryl trioleate hydrolase activities of H-TGL and LPL were measured under the conditions optimal for these enzymes (see Methods section). For LPL, serum (60 μl/ml of assay medium) was added as the source of activator apolipoprotein. Concentrations of palmitoyl-CoA and enzyme in the assay media were ⅛ those of the concentrations during preincubation. Final assay volume was 0.46 ml.

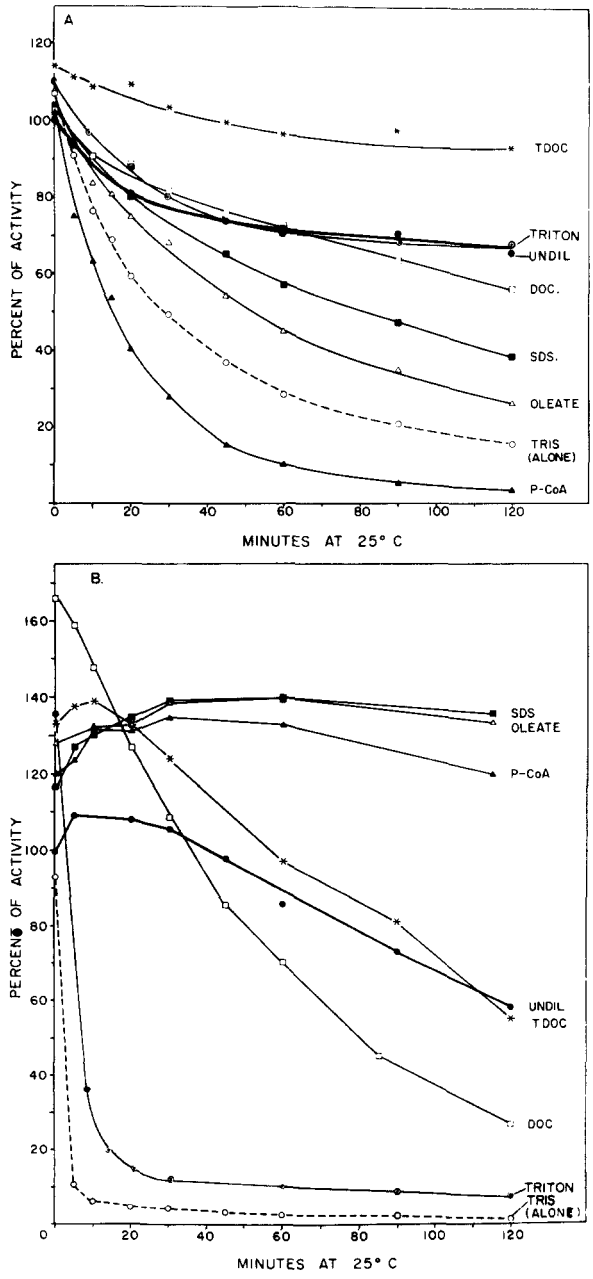
the 2 hr of pre-incubation in the presence of palmitoyl-CoA, Tris-oleate, or sodium dodecyl sulfate. On the other hand, LPL diluted in 0.8 mM Triton X-100, which also gave activities higher than the undiluted control, when assayed 45 sec after dilution, lost activity rapidly upon pre-incubation. This was also true when different concentrations of Triton X-100 (0.015–1.0 mM) were used. Conversely, activity of LPL in the presence of sodium deoxycholate was better retained when the detergent concentration was lowered to 1/5 that used in the experiment of Figure 6B.

Not shown in Figure 6B are data obtained after pre-incubation of LPL at 25°C for longer periods of time. In contrast to the rapid decrease of LPL activity, after 2 hr of pre-incubation in buffers containing palmitoyl-CoA (possibly due to enzymatic hydrolysis of the acyl derivative), LPL activity was 80% retained in the presence of either sodium dodecyl sulfate or Tris-oleate, even after pre-incubation for 144 hr at 25°C. Of special interest was the finding that, after pre-incubation for 5 min at 26°C with 1 mM sodium dodecyl sulfate (0.125 mM in the assay medium), the glyceryl trioleate hydrolase activity of LPL was 122% that of the undiluted control (137% activity was obtained with LPL pre-incubated with 0.4 mM sodium dodecyl sulfate under the same conditions) while H-TGL was almost completely inactivated (remaining glyceryl trioleate hydrolase activity was 1.4% that of the undiluted control). Sodium decyl sulfate was less effective than the dodecyl salt in preserving LPL activity or in inhibiting H-TGL. Pre-incubation of these enzymes with 1 mM sodium dodecyl sulfate did not eliminate the requirement of LPL for apoC-II, nor did it produce a requirement for a serum cofactor for H-TGL activity, as shown by the similar results obtained with this enzyme in assay media with or without serum. In an experiment similar to that described in Table 2 for pre-incubation mixtures without albumin, egg lysolecithin (0.005–2.0 mM) enhanced glyceryl trioleate hydrolase activity of H-TGL, while having very little protective effect on this activity of LPL. Maximal values with H-TGL approximating 115%, as compared with the undiluted control, were obtained with 0.2–0.5 mM lysolecithin. Protection of the glyceryl trioleate hydrolase activity of H-TGL by lysolecithin was sustained for an extended period of time. Thus, H-TGL diluted in Tris buffer containing 0.3 mM lysolecithin retained, after 24 hr of pre-incubation at 25°C, 85% of its activity (as compared with the value obtained with the undiluted, nonincubated

control). Only 23% of the activity of the undiluted control and none of the activity of H-TGL diluted in Tris buffer without lysolecithin were measurable after pre-incubation for 24 hr at 25°C.

Comparison of the rate of inactivation of the two hydrolase activities of H-TGL and of LPL

The experiments in **Fig. 7** provide further support to the hypothesis that the two hydrolytic activities observed with both H-TGL and LPL are associated with each enzyme. The enzyme preparations were pre-incubated at 25°C at the concentration of buffers as obtained from the heparin–Sepharose column, or after dilution of these eluates with Tris-HCl buffer. At the times indicated, samples were assayed for remaining activity using both substrates. Results with H-TGL (Fig. 7A) show that the glyceryl trioleate and palmitoyl-CoA hydrolase activities, of both diluted and undiluted enzyme, decreased in parallel. Using LPL (Fig. 7B) and undiluted enzyme, simultaneous decreases in the two hydrolytic activities with time were also observed. However, glyceryl trioleate hydrolase activity fell more rapidly than the palmitoyl-CoA hydrolase activity with the diluted sample. Inactivation of LPL by dilution (with a buffer without NaCl) is probably preceeded by aggregation, and this aggregation can be partially reversed by addition of a detergent (palmitoyl-CoA in this case). Therefore, the results are consistent with the postulate that both H-TGL and LPL are active toward both substrates.



DISCUSSION

Evidence has been presented for the hydrolysis of glyceryl trioleate and palmitoyl-CoA by both H-TGL and LPL purified from postheparin plasma. Thus, both hydrolase activities cochromatographed with both enzymes (Fig. 1), the levels of these two activities showed a strong correlation in preparations of H-TGL and LPL purified from 15 normal subjects (Fig. 2), and both hydrolase activities in the two enzymes decreased in parallel on pre-incubation at 25°C (Fig. 7). A similar conclusion was reached by Jansen and Hülsmann (28–30) for these enzymes from rat postheparin plasma. However, our data differ from those of Jansen and Hülsmann in that we find with H-TGL higher rates of hydrolysis of glyceryl trioleate than of palmitoyl-CoA.

Fig. 6. Effects of detergents on stability of H-TGL and LPL at 25°C. H-TGL and LPL (as obtained from the Heparin–Sepharose column) were diluted 6- and 3-fold, respectively, in buffers with or without detergents to obtain a final concentration of 40 μg of enzyme protein/ml. At the times indicated, aliquots of the incubation mixture were tested for glyceryl trioleate hydrolase activity using the assay conditions described in the legend of Table 2. All components were readily soluble in the buffer, except oleic acid, which was sonicated into the buffer until a clear solution was obtained. Time curves represent effect of pre-incubation of undiluted enzymes (● —— ●) or, the enzymes diluted in 0.2 M Tris-HCl buffer, pH 8.2, alone (○ – – – ○) or containing detergents as indicated: 0.4 mM oleic (△), 0.2 mM palmitoyl-CoA (▲), 5 mM sodium deoxycholate (□), 0.4 mM sodium dodecyl sulfate (■), 0.5 mM sodium taurodeoxycholate (*), or 0.8 mM Triton X-100 (⊙). Activities are expressed relative to the activity of the undiluted enzymes assayed immediately after thawing. Figure 6A, H-TGL; Figure 6B, LPL.

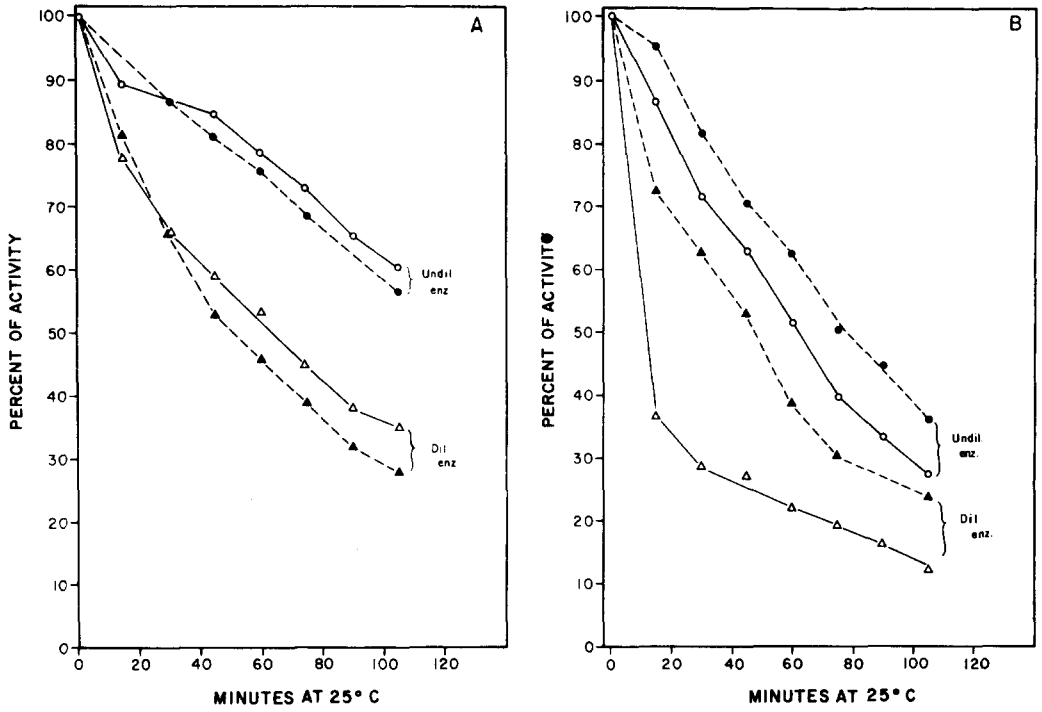


Fig. 7. Time course of inactivation of glyceryl trioleate and palmitoyl-CoA hydrolase activities of H-TGL and LPL. Preparations of H-TGL (Fig. 7A) and LPL (Fig. 7B) were incubated at 25°C either at the concentration of the heparin–Sepharose column eluates (242 and 132 μg of protein/ml for H-TGL and LPL, respectively) (○,●) or after dilution with 0.1 M Tris-HCl–1 mM EDTA buffer, pH 8.5 (to a protein concentration of 50 μg/ml) (△,▲). Pre-incubated samples were then assayed for glyceryl trioleate hydrolase activity (open symbols) or palmitoyl-CoA hydrolase activity (closed symbols), as described in the Methods section. Activities are expressed relative to those obtained without pre-incubation. (The absolute values of these activities, expressed in μmol of FFA or CoA liberated/hr per ml of enzyme, were: 39.1 μmol FFA and 35.7 μmol CoA for undiluted H-TGL; 34.9 μmol FFA and 29.7 μmol CoA for diluted H-TGL; 14.2 μmol FFA and 2.5 μmol CoA for undiluted LPL; and 1.39 μmol FFA and 0.62 μmol CoA for diluted LPL.)

The data of Fig. 3A and Table 1 shows that albumin can account for all of the activating effects of serum reported previously for the hydrolysis of palmitoyl-CoA by rat postheparin plasma (28). The increased linearity of the rates of palmitoyl-CoA hydrolysis by either H-TGL or LPL observed in the presence of albumin is consistent with the known action of this protein to bind free fatty acids and to protect enzymes from denaturation in dilute solutions. However, albumin was still required when H-TGL was diluted to levels that produced hydrolysis of palmitoyl-CoA to the same degree as that obtained with LPL (in the absence of albumin), in the same length of time (data not shown). Thus, the difference between H-TGL and LPL (Fig. 3), when measuring palmitoyl-CoA hydrolysis in a medium without serum or albumin, could not be explained by inhibition of H-TGL by the greater amount of free palmitic acid accumulated in the assay medium.

Reports in the literature show that palmitoyl-CoA, which is known to have detergent properties, causes inhibition of some enzymes while not affecting others (31). The inhibitory effect of the high levels of palmitoyl-CoA in the assay medium is suppressed by addition of albumin or other proteins (i.e., heat-denatured liver microsomes) (31, 46) which probably bind the acyl derivative. Similarly, it is likely that, in the H-TGL assay system, albumin or serum is required to decrease the concentration of "free palmitoyl-CoA", which inactivates the enzyme at concentrations above 50 μM (Table 2). The apparent stimulatory effect of serum in assay systems measuring hydrolysis of palmitoyl-CoA by H-TGL (Fig. 3A) can, hence, be explained through this protective mechanism. The requirement for albumin by this enzyme, in the hydrolysis of glyceryl trioleate, is explained by a different mechanism, i.e., the removal of fatty acids produced during the reaction (Fig. 5). In this system, containing the optimal amount of albumin, addition of serum proved to be inhibitory. This inhibition was variable (dependent upon the serum added) and has not been fully explained. Inhibition of H-TGL by

larger amounts of serum than those used in the experiments of Fig. 1 and by the purified apoC peptides has been reported recently (47).

Tris-oleate, like the potassium salts of oleic, linoleic, and linolenic acids (1, 9, 43), is shown here to protect LPL and, to a lesser degree, H-TGL. The resumption of hydrolysis by addition of albumin to an assay mixture without it (Fig. 5) is also consistent with the postulate that free fatty acids in the assay medium do not inactivate the enzyme, but inhibit the further hydrolysis of substrate (product feedback inhibition). This effect is somewhat more pronounced for H-TGL than for LPL. Presently under investigation is the postulate that H-TGL, like the activities in rat liver microsomes (46) and rat liver plasma membranes (48), is primarily an acyltransferase, which also has hydrolytic activity. If hydrolysis of glyceryl trioleate or palmitoyl-CoA produces the substrate for a transferase reaction, the hydrolase activity should be suppressed in the absence of an acceptor of the free fatty acids produced. On the other hand LPL, primarily as a hydrolase, would be less affected by product accumulation.

Triton X-100 has been used as emulsifying agent for glyceryl trioleate in assay systems measuring H-TGL and LPL from different sources (12, 49). The findings presented here that Triton X-100 affects the two enzymes differently raises a question regarding the validity of such assays. However, unpublished experiments from this laboratory gave very similar values when purified H-TGL and LPL were assayed in the system described by Krauss, Levy, and Fredrickson (49) and in the gum arabic system used in the current work. The higher levels of albumin and glyceryl trioleate in the assay system of Krauss et al. (49) do protect the enzymes from inhibition by the detergent.

Protection and activation of another lipolytic enzyme, porcine pancreatic lipase, by detergents (bile salts) has been the subject of several recent studies (50–53). Porcine pancreatic lipase and LPL also share a number of other characteristics: *a*) both enzymes are glycoproteins that are activated by small molecular weight polypeptides (colipase for pancreatic lipase) (51, 52, 54) and apoC-II for LPL (11, 12); *b*) both have similar substrate specificities, including activity toward palmitoyl-CoA (55); and *c*) the sodium chloride concentration in the assay media affects LPL and pancreatic lipase identically, provided that the latter enzyme is assayed in the presence of bile salts (51, 52, 54, 56, 57). It is possible, therefore, that detergents protect and activate LPL not only by preventing aggregation, but also by mechanisms similar to those summarized by Brockerhoff and Jensen (54) for the effect of bile salts on pancreatic lipase, including protection against the denaturation caused by unfolding at the air–water interface and absorption and denaturation at the oil–water interface. Thus, detergents prevented the inactivation of LPL by dilution, as shown in Figs. 4 and 6. LPL also strongly absorbs to the oil–water interface (1, 9) as does pancreatic lipase (58, 59), and apoC-II may bind to both enzyme and substrate, as colipase binds to the pancreatic lipase–sodium taurodeoxycholate complex. Unlike pancreatic lipase, which has substantial activity even in the absence of colipase (51), LPL has only very low activity in the absence of apoC-II. Albumin cannot substitute for this cofactor under any of the conditions tested with LPL, while albumin can partially replace the requirement of pancreatic lipase for colipase in the presence of detergents (51).

In this work the amounts of detergents used are expressed in molar concentrations and the results presented apply to the specified set of conditions. One should be aware, however, that the weight ratio of detergent to protein, the ionic strength and the pH of the medium, as well as the time of equilibration of the protein with the detergent, are important factors in obtaining a given effect (60–62). Indeed, when postheparin plasma was used instead of purified H-TGL, 50 mM or higher concentrations of SDS were required to obtain the same degree of inhibition as that observed with 1 mM SDS and the purified enzyme (data not shown).

In the experiments presented in this work the comparison of the effect of the several detergents on purified H-TGL and LPL was done maintaining the same detergent to protein ratio and the same pH. The higher inhibition of H-TGL by SDS cannot be explained by differences in the ionic strength during pre-incubation. It has been reported that higher NaCl concentrations facilitate binding of SDS to protein (61); however, the NaCl concentration was higher in the mixtures for LPL, which was not inhibited by SDS.

Pancreatic lipase has been shown to be inhibited by SDS (50, 51, 53). However, higher detergent to enzyme protein ratios and more prolonged preincubation periods were used in those experiments than in this work. This makes difficult any comparison of the sensitivity of LPL and pancreatic lipase to the effects of SDS.

Most proteins bind SDS, and a large and relatively constant quantity of SDS is found to bind per gram of protein (1.1–2.2 g SDS per g of protein) (61, 63). The binding of these large amounts of this detergent results in denaturation of the protein and consequent loss of activity if the protein is an enzyme. Some enzymes have been reported to be resistant to SDS binding and, therefore, to the concurrent inactivation (i.e., glucose oxidase, pepsin, and papain

(61)). Retention of trypsin and chymotrypsin proteolytic activities in sodium dodecyl sulfate solution (64, 65) and a SDS-stable form of gramicidin S synthetase (66) have also been reported recently. Except for the latter communication, where the concentrations of SDS used are not given, the enzymes are resistant to detergent concentrations much larger than those used in this work. It is very possible that protection of LPL by SDS is not due to lack of binding of SDS, since larger concentrations of SDS are inhibitory. Most likely, SDS protects LPL in a manner similar to the protective effect on proteins, by *low* concentrations of SDS, reported by others, i.e., protection of albumin from heat coagulation or denaturation by urea or guanidine hydrochloride and stabilization of β-lactoglobulin against heat and alkali (in ref. 60).

In the absence of detergents, LPL is a much more labile enzyme than H-TGL. Whether this is the result of the higher degree of purity of the LPL, or due to the differences in the sugar content of the two enzymes (37) or to some other causes cannot be evaluated at the present time. The diverse effect of the same detergent on H-TGL and LPL shows further dissimilarities between these two enzymes. It is of interest to note that the detergents most effective in protecting LPL from inactivation are amphiphiles with long chain hydrocarbon tails and are thus analogous to its natural substrates. How this relates to the enzymatic activity in vivo needs further investigation. Last, but not least, the finding that incubation with sodium dodecyl sulfate results in complete inactivation of purified H-TGL, with only a minimal loss of activity of purified LPL, may provide the basis for an assay system in which LPL can be determined directly in the presence of H-TGL.

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