Characterization, Subcellular Localization, and Partial Purification of a Heparin-released Triglyceride Lipase from Rat Liver*

GERD ASSMANN, RONALD M. KRAUSS, DONALD S. FREDRICKSON, AND ROBERT I. LEVY

From the Molecular Disease Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

Post-heparin plasma in the rat contains triglyceride lipase activity of both hepatic and extrahepatic origin. The lipase released into rat hepatic perfusate by heparin has been characterized in an assay containing Triton X-100, albumin, and [14C]triolein. Fatty acid release was linear for 120 min over a wide range of enzyme concentrations at 27° and 37°. The pH optimum was 9.5. Enzymatic activity was >80% inhibited by prior incubation with 0.5 M NaCl or 750 µg of protamine per ml. The apparent $K_m$ was 1.28 mM. The enzyme was localized in rat liver cell fractions. Cell fractions were checked for purity by marker enzymes and electron microscopy. Activity inhibited by NaCl or protamine was found in plasma membranes, microsomes, and cytosol, having pH optima of 9.5, 9.0, and 8.0, respectively. A different triglyceride lipase not affected by NaCl or protamine and having a pH optimum at 4.4 was found in lysosomes. The plasma membrane-bound lipase had a specific activity 10 times that in liver homogenate. Addition of heparin to the plasma membrane fractions in a concentration as low as 1 unit per ml effected the release of the enzyme into the medium. The plasma membrane-released lipase had the same apparent $K_m$. A 360-fold enhancement of plasma membrane lipase activity over that in whole liver homogenate was achieved by heparin affinity chromatography.

EXPERIMENTAL PROCEDURE

Materials

Glyceryl tri-[1-14C]oleate and [2-3H]glyceryl trioleate (Amersham-Searle) were >98% pure as judged by thin layer chromatography (Silica Gel G, solvent system: petroleum ether-ether-acetic acid, 90:10:1); other chemicals were obtained from the following sources: unlabeled triolein (Applied Science); fatty acid-free albumin (Pentex); Triton X-100 (Packard); Triton WR 1339 (Serva, Heidelberg); glucose 6-phosphate, 5'AMP (Boehringer); cytochrome c from horse heart, type III (Sigma); p-nitrophenylphosphate (Merck); heparin (sodium) 20,000 U.S.P. units per ml (Upjohn Company); protamine sulfate (saline) (Calbiochem); bovine serum albumin (fraction V, Armour); Bio-Gel P-200 (Bio-Rad).

The pH optima were determined with the following buffers: (ionic strength, $I = 0.1$): sodium citrate buffer, pH 3.0, pH 3.4; sodium acetate buffer, pH 3.8, pH 4.4, pH 5.0, pH 5.6; sodium phosphate buffer, pH 6.6, pH 7.4, pH 8.0; glycine-NaOH buffer, pH 8.6, pH 9.0, pH 9.4, pH 9.5, pH 9.6, pH 10.0, pH 10.4. All chemicals, including buffers, were "analytical" or "certified" grade.

Methods

Rat Liver Perfusion—Isolated livers from fed 400-g male N.I.H. Osborn-Mendel rats were perfused as described previously (22). The perfusate consisted of a 1:1 mixture of defibrinated rat blood and Krebs-Ringer bicarbonate buffer containing 3% bovine serum albumin and 0.1% glucose and adjusted to pH 7.4 with NaCO$_3$. After initiation of perfusion, 2500 units of heparin were added (50 units per ml) and the recycled post-heparin perfusate, in a total volume of about 50 ml, was collected 15 min later. It was initially determined that triglyceride lipase activity was not present in the perfusate.
before heparin was introduced and that heparin released lipolytic activity within 2 min, the maximum being reached by 15 min (23). Aliquots were stored at $-20^\circ$ and were stable for at least 6 months.

**Preparation of Subcellular Fractions**—Livers were homogenized with a Dounce homogenizer and fractionated into nuclear, cytoplasmic, mitochondrial, light mitochondrial, microsomal, and supernatant fractions according to the method of deDuve et al. (24). Lysosomes were prepared according to the procedure of Wattiaux et al. (25). Triton WR 1339 in 0.9% NaCl solution, 1.7 mg per g of body weight, was injected into the tail vein of adult rats weighing about 150 g. After 90 hours the animals were killed, and the livers were quickly removed and minced with scissors. The tissue was homogenized in 8 volumes of 0.25 M sucrose, and the “mitochondrial fraction” was separated by centrifugation over a continuous sucrose gradient (0.8 to 1.6 M) into lysosomes and mitochondria. Plasma membranes were obtained from crude nuclear fractions by the method of Neville (36) as modified by Ray (27). The membranes were collected from the discontinuous sucrose gradients at the 37% to 41% (w/w) interface, washed twice with 1 mM NaHCO$_3$, 0.5 mM Ca$^{2+}$ solution and the final pellet taken up in the same buffer solution (usually 5 mg of protein per ml). The yield was 0.5 to 0.75 mg of protein per g of fresh rat liver.

**Analytical Procedures**—In all preparations, the following determinations were performed on each cell fraction: 5'-nucleotidase (28), glucose 6-phosphatase (29), acid phosphatase (30), cytochrome c oxidase (31, 32), protein (33). Inorganic phosphate was measured by the method of Chen (34). Enzyme activities were expressed as micromole of substrate utilized per mg of protein per min.

**Assay of Triglyceride Lipase Activity**—Lipase activity was determined by the method of Krauss et al. (35). Components of the assay and their final concentrations were: glycerol tri-[1-14C]oleate, 10.5 nM; unlabeled triolein, 8.46 μM; fatty acid-free albumin, 15 mg per ml; and Triton X-100, 4.5 × 10$^{-4}$ M per ml. In the usual assay these constituents were sonicated in glycine-NaOH buffer (I = 0.1, pH 9.5) for exactly 60 s at 60 W and 4° using the mcerat of a Bronson sonifier (Heat Systems, Inc., model W185). All assays were performed in duplicate and carried out in a Dubnoff shaking water bath at 27° or 37° for 1 to 2 hours. The fatty acids were then extracted from 1.0 ml of the incubation mixture by the method of Schots et al. (35) and counted in a Packard model 7201. [2-3H]Glycerol was separated by extrac-

**Characterization of Hepatic Cell Fractions**—The purity of the cell fractions was monitored by both enzymatic measurements and electron microscopy. The plasma membrane and endoplasmic reticulum fractions appeared to be about 10% cross-contaminated according to their content of glucose 6-phosphatase and 5'-nucleotidase, respectively (Table I). Lysosomes prepared by continuous gradient were free of mitochondria. The mitochondrial fraction contained acid phosphatase and cytochrome c oxidase activities suggesting <2% contamination with lysosomes. The specific activity of the marker enzyme, 5'-nucleotidase, in the plasma membrane preparations was enriched 16.3-fold over that in the whole homogenate. In electron micrographs the plasma membranes were vesicular and contained junctional complexes; only an occasional patch of rough endoplasmic reticulum was seen.

**Optimization of Lipase Assay Conditions**—The addition of albumin and Triton X-100 to the incubation medium was necessary for maximal rates of triglyceride hydrolysis by heparin-releasable hepatic lipase (Table I1). The hydrolysis of triolein by liver perfusate and liver cell fractions over a range of protein concentrations of 10 μg to 1000 μg per ml proceeded linearly for 120 min at both 27° and 37°. The rate of free fatty acid release by the post-heparin liver perfusate was independent of heparrin
hydrolysis catalyzed by lipase in the different cell fractions. Approximately 0.25% of the triglyceride was hydrolyzed to glycerol and free fatty acids; approximately 0.25% was sonified in the presence of different amounts of Triton X-100 or albumin. Values are expressed in percentage of maximal hydrolytic activity as determined by free fatty acid release.

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<tr>
<td>3.0</td>
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Tables I and II: Determination of marker enzymes in different cell fractions and Effect of different concentrations of Triton X-100 and albumin in emulsification mixture on free fatty acid release.

The stoichiometry of [14C]fatty acid and [3H]glycerol release was investigated using glyceryl-[1-14C]trioleate and [2-3H]glyceryl trioleate as substrates. The 3H:3C ratios in di- and mono-glycerides and the radioactivity individually present as [2-3H]glycerol and [14C]fatty acids were determined. When the post-heparin perfusate was used as enzyme source and after incubation at 27°C for 60 min, 5% of the triglyceride was hydrolyzed to glycerol and free fatty acids; approximately 0.25% was sonified in the presence of different amounts of Triton X-100 or albumin. Values are expressed in percentage of maximal hydrolytic activity as determined by free fatty acid release.

The pH optimum of the triglyceride lipase activities in these fractions were 8.0, 9.0, and 9.5, respectively. At pH 9.5, the activity in the cytosol was 80% of that obtained at pH 8.0. The specific activities of the cell fractions at pH 9.5 are shown in Table III. When corrected for contamination based on measurements of the marker enzymes (Table I), the specific activity of the lipase was about 8-fold greater in the plasma membranes than in cytosol and microsomes. The bulk of the cell protein is present in cytosol, and much higher specific activity released from the plasma membranes in the alkaline pH range is probably located in this cell fraction. It is not possible, however, to measure the amount of possible contamination of the cytosol with lipase of much higher specific activity released from the plasma membranes during cell fractionation.

We were unable to demonstrate significant lipase activity in mitochondria. Lipase activity at acid pH paralleled the distribution of acid phosphatase throughout the fractionation procedure and appeared to be exclusively of lysosomal origin.
lipase activity is normally released into the post-heparin perfusate at pH 7.4. Investigation of the release of lipase activity by heparin in vitro was also carried out at this pH. Plasma membranes obtained from the discontinuous sucrose gradient were washed twice with a solution containing 1 mM NaHCO₃ and 0.5 mM Ca++ and the final pellet was resuspended in the same medium at a protein concentration of 5 mg per ml. As shown in Fig. 2, the addition of small amounts of heparin to the plasma membrane suspension caused the release of significant amounts of hydrolytic activity into the incubation medium. Prior incubation with heparin slightly increased the total lipase activity in the suspension of plasma membranes.

When the membranes were separated subsequently by centrifugation it was apparent that heparin had liberated approximately 85% of the membrane lipase activity into the supernatant (Table IV). The triglyceride lipase released from plasma membranes by heparin had the same pH optimum (pH 9.5) as the lipase in post-heparin liver perfusate (Fig. 1).

**Effect of Substrate Concentration, NaCl, and Protamine on Triglyceride Lipase in Liver Perfusate and Plasma Membranes**

The effect of substrate concentration on the rate of hydrolysis is shown in Fig. 3. A $K_m$ of 1.28 mM was obtained from the Lineweaver-Burk plot for the lipase in post-heparin perfusate and the enzyme released from isolated plasma membranes.

The activity of triglyceride lipase in the post-heparin hepatic perfusate and liver cell fractions after preincubation with NaCl, over a wide range of concentrations, is shown in Table V. Maximum activity was usually obtained in the absence of added NaCl, but prior incubation of the liver perfusate and microsomes with very high NaCl concentrations (2.5 x and 3.75 x) augmented enzymatic activity above the base-line. The lipase activities in microsomes, cytosol, liver perfusate, and that released from the plasma membranes were inhibited from 80 to 90% by prior incubation in NaCl at concentrations of 0.31 or 0.63 M. The acid lysosomal lipase activity was inhibited less than 10% at the same salt concentrations. At NaCl concentrations greater than 0.63 M, lipases from different sources other than lysosomes showed a progressive increase in activity. The increase in lipase activity at higher salt concentrations has been previously observed (1) and remains unexplained.

The effects of increasing protamine sulfate concentration on the several lipase activities are illustrated in Table VI. There was no appreciable inhibition of lysosomal triglyceride lipase, but small concentrations of protamine reduced the activity of the other enzyme preparations by 60 to 85%. The degree of inactivation was dependent upon the duration of prior incubation with the inhibitor (Table VII). The concentration of

![Fig. 2. Effect of heparin on the release of triglyceride lipase from plasma membranes. Plasma membranes were incubated in a 1 mM NaHCO₃, 0.5 mM Ca++ solution for 15 min at 27° in the presence of different heparin concentrations. Incubation volume was 1 ml. Subsequently, plasma membranes were sedimented for 15 min (4°) at 50,000 x g and 100-μl aliquots of the supernatant and the plasma membranes (resuspended to the original concentration) determined for lipolytic activity (incubation for 120 min at 37°). ▲▲▲, lipolytic activity in the supernatant; 0- - -0- - -0, represents residual lipolytic activity in plasma membranes. FFA, free fatty acid.](http://www.jbc.org/)

![Fig. 3. Reciprocal plot of reaction velocity (expressed in counts per min of [1-14C]free fatty acid released per 90 min) versus triolein concentration.](http://www.jbc.org/)

**Figure 3.** Reciprocal plot of reaction velocity (expressed in counts per min of [1-14C]free fatty acid released per 90 min) versus triolein concentration. Triolein (specific activity = 0.11 μCi per μg) was emulsified in the presence of 100 mg of albumin and 5 mg of Triton X-100 in 6 ml of glycine buffer (I = 0.1), pH 9.5. The final incubation volume of 1 ml contained 25 μl of post-heparin perfusate and 100 μg of heparin-released plasma membrane protein plus 25 μl of pre-heparin perfusate, respectively. Incubations were done for 90 min at 27°. All determinations were performed in duplicate. $K_m = 1.28 \times 10^{-3} \text{M}$. TGL, triglyceride lipase.

### Table IV

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<th>Aliquot</th>
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<tr>
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</tr>
<tr>
<td>4</td>
<td>Plasma membranes</td>
</tr>
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<td>Supernatant</td>
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Plasma membranes suspended in 1 mM NaHCO₃, 0.5 mM Ca++ (protein concentration 5 mg per ml) were divided in five aliquots. Aliquot 1 was preincubated for 15 min at 27° without further treatment. Aliquot 2 was preincubated for 15 min at 27° in the presence of 20 units of heparin per ml. Aliquot 3 was treated in the same way as Aliquot 1 except that after the end of preincubation plasma membranes and supernatant were separated by centrifugation (15 min; 4°; 50,000 x g); the plasma membrane pellet was resuspended with incubation to the original concentration. Aliquot 4 was treated in the same way as Aliquot 2 except that after the end of preincubation 20 units of heparin were added to the supernatant, preincubation was continued at 27° for further 5 min. One hundred microliters of each aliquot were taken at the end of preincubation respective centrifugation and incubation continued for 120 min at 37° by adding 0.9 ml of substrate. Each number is the mean of two experiments (four determinations).
Inhibition of post-heparin liver perfusate triglyceride lipase and different cell fractions with sodium chloride

Aliquots of the different enzyme sources were preincubated for 60 min at 27°C in a total volume of 0.2 ml with glycine buffer (I = 0.1), pH 9.5, containing the indicated concentrations of sodium chloride. Following preincubation, a 0.05-ml aliquot was taken and adjusted to a concentration of 2.0 M NaCl in a volume of 0.1 ml. Then 0.9 ml of substrate mixture containing no salt was added so that the final salt concentration was 0.20 M, and incubation was continued for 60 min at 37°C. Inhibition of lysosomal triglyceride lipase was determined at pH 4.4 (acetate buffer, I = 0.1).

<table>
<thead>
<tr>
<th>Sodium chloride (M)</th>
<th>Post-heparin liver perfusate</th>
<th>Heparin-released plasma membrane triglyceride lipase</th>
<th>Microsomes</th>
<th>Cytosol (100,000 x g supernatant)</th>
<th>Lysosomes</th>
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% maximal activity

Inhibition of lipolytic activity of post-heparin liver perfusate and different cell fractions with protamine

Aliquots of the different enzyme sources were incubated for 60 min at 27°C in a total volume of 0.2 ml with glycine buffer (I = 0.1), pH 9.5, containing the indicated concentrations of protamine. Following preincubation, a 0.05-ml aliquot was taken, adjusted to 0.1 ml with glycine buffer and incubation continued for 60 min at 37°C. Inhibition of lysosomal triglyceride lipase was determined at pH 4.4 (acetate buffer, I = 0.1).

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<th>Protamine concentration (μg/ml preincubation)</th>
<th>Post-heparin liver perfusate</th>
<th>Plasma membranes</th>
<th>Heparin-released plasma membrane triglyceride lipase</th>
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<th>Cytosol (100,000 x g supernatant)</th>
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% maximal activity

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Hydrolytic activity of different fractions

Effect of time of preincubation with protamine on inhibition of post-heparin liver perfusate and different cell fractions

Aliquots of indicated enzyme sources were incubated at varying time intervals and 27°C in a total volume of 0.4 ml of glycine buffer, I = 0.1, pH 9.5, containing 1500 μg per ml of protamine. After the indicated time period, 0.1-ml aliquots were taken and incubation continued for 60 min at 37°C by adding 0.9 ml of substrate mixture. Inhibition of lysosomal triglyceride lipase was tested at pH 4.4 (acetate buffer, I = 0.1).

Further Purification of Plasma Membrane Triglyceride Lipase—

The plasma membrane triglyceride lipase was partially purified by chromatography on the heparin affinity column. This column did not bind any of the lipase released from the membranes by heparin; all of this activity emerged in the void volume. Therefore, advantage was taken of the observation that enzyme activity could be released from the membranes in the absence of heparin (Table IV). From 30 to 50% of the lipase activity released by heparin was obtained by incubation in glycine buffer alone. Up to 80% of this enzymatic activity was bound to the column.1 About half of this activity was subsequently eluted by increasing salt concentrations, the maximal activity being obtained at conductivity 32 mmho (Fig. 4). This chromatographic step resulted in a 15- to 20-fold increase in specific activity (Table VIII). The degree of purification as observed by sodium dodecyl sulfate gel electrophoresis is illustrated in Fig. 5. Sodium dodecyl sulfate or urea was required for satisfactory separation of the plasma membrane proteins. The lipolytic activity of the purified fraction was destroyed by 5 min prior incubation at 4°C with either of these components, and it was not possible to locate the position of the enzyme protein on polyacrylamide gel.

The activity of the partially purified fraction was not enhanced by the addition of rat plasma (0.04 ml per ml of assay), which contains an activator of lipoprotein lipase (3).

**DISCUSSION**

We have further characterized the triglyceride lipase released by heparin from the perfused rat liver and have attempted to identify its subcellular origin. The heparin-released hepatic

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1 We have recently used a column in which heparin has been covalently linked to agarose according to the method of Cuatrecasas (44). The enzyme released from plasma membranes was completely bound to this column and could subsequently be released by increasing salt concentration.
lipase has a pH optimum at 9.5, an apparent $K_m$ of 1.28 mm, and is inhibited by prior incubation with protamine and salt. Of the hepatic cellular fractions studied, only the plasma membranes contained triglyceride lipase activity with a pH optimum of 9.5. The lipolytic activity in isolated plasma membranes proved to be readily displaced by heparin, more than 85% being released during 15 min incubation in the presence of very small amounts of this substance. The enzyme activity had the same pH optimum, the same response to inhibitors, and the same $K_m$ as the lipase released from the perfused liver.

When plasma membranes were incubated in the absence of heparin, only 25% of their total lipase activity was released into the medium. Addition of heparin to the enzyme released in this fashion did not increase its activity. The nonspecific release of lipase activity may be due to solubilization of some membrane proteins by the alkaline incubation medium.

The specific activity of lipase having a pH optimum at 9.5 was 9- to 10-fold higher in the plasma membranes than in liver homogenate and about 8-fold higher than that in cytosol and microsomes. The lipase activity in cytosol and microsomes was inhibited by NaCl and protamine. These inhibition studies, as well as other characterization of enzyme activity in different cell fractions, were carried out at pH 9.5, the pH optimum of lipase in liver perfusate. This pH is higher than the optimum observed in cytosol and microsomes, although at pH 9.5 activity in these two-cell fractions is more than 80% of the maximum.

We were unable to determine to what extent the lipase activity in cytosol and microsomes represented contamination with enzyme located in plasma membranes. We observed release of some activity when plasma membranes were incubated with the buffer (pH 7.4) used throughout the homogenization and fractionation procedure. On the other hand, if the activities of triglyceride lipase and 5'-nucleotidase in plasma membranes were not affected differently by the homogenization and fractionation procedure, it is not possible to attribute all of the lipolytic activity at pH 9.5 in cytosol and microsomes to enzyme originating in the plasma membranes.

Lipase activity in plasma membranes has been described by Higgins and Greten (21) who prepared plasma membranes according to the method of Wallach and Ullrey (41), and by Guder et al. (16), who employed the method of Coleman (42) with omission of the final gradient step. The enzyme described by Higgins and Greten had a pH optimum in the acid range and appeared to be activated by heparin. The activity found by Guder et al. had a pH optimum of 7.6 and was activated, but not shown to be released, by heparin.

Stoffel and Greten first reported that acid triglyceride lipase activity was mainly localized in lysosomes (13), and these results have been confirmed by other authors (14-17). The present studies show that acid lipase of lysosomal origin was inhibited less than 10% by sodium chloride or protamine concentrations that inhibited alkaline lipase activity in other subcellular fractions.
fractions by >75%. Our data differ from the results of Mahadevan and Tappel (15) who found no effect of protamine on lysosomal lipase activity but observed a 50% inhibition with 0.5 M sodium chloride. These differences may be related to their use of unlabeled glycerol ester as substrate and a different emulsification procedure.

There have been conflicting reports on the existence of a triacylglyceride lipase in mitochondria. Purification of a lipase from rat liver mitochondria has recently been reported (19), although the purity of the cell fractions was not established unequivocally. We observed no lipolytic activity in mitochondria, either in the acid or alkaline range. These mitochondria had a high degree of purity as established by marker enzymes and electron microscopy. It is conceivable that mitochondrial lipase activity could have been inhibited by Triton WR-1339. It has been shown, however, that this detergent is not taken up by hepatic mitochondria (41). Our data are consistent with the results of other authors who found that lipolytic activity in the light mitochondrial fraction followed the distribution patterns of marker enzymes of lysosomes rather than mitochondria (16).

Tissue lipases are considered to be of major importance for the metabolism of plasma triglycerides. The existence of lipolytic activity against chylomicrons in liver has first been demonstrated by Korn (2). His criteria for defining lipoprotein lipase rested, in part, on its activation by heparin and sensitivity to protamine and salt inhibition, and partly on a requirement for a serum component for its optimum activity. This serum component has been lately identified as one of the apolipoproteins for a serum component for its optimum activity. This serum protein lipase in extrahepatic tissues. Furthermore, it is also not possible to distinguish between them in a functional sense. The plasma membrane enzyme appears to be the major component of lipolytic activity released by heparin into hepatic perfusate. These perfusates contain activity capable of hydrolyzing triglycerides in lipoproteins. It has been recently suggested that “remnants” of lipoproteins are the major substrates for triglyceride lipase. Lipolytic activity at the plasma membrane may conceivably facilitate the further catabolism of lipoproteins by promoting their delipidation prior to entry into the hepatic cells.

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Characterization, Subcellular Localization, and Partial Purification of a Heparin-released Triglyceride Lipase from Rat Liver
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