Hydrolysis of Triolein in Phospholipid Vesicles and Microemulsions by a Purified Rat Liver Acid Lipase*

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An acid lipase was purified from rat liver lysosomes. Lipase purification involved affinity chromatography, gel filtration, and stabilization of the purified preparation using ethylene glycol and Triton X-100. A molecular weight of 67,000-69,000 was determined independently using density gradient centrifugation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and gel filtration. To study enzyme action, model substrates were prepared by incorporating radiolabeled triolein into either unilamellar vesicles or microemulsions. Substrates were prepared by cosonicating aqueous dispersions of lecithin and triolein. Formation of vesicles or emulsions depended on the relative amount of each lipid and on sonication conditions. Vesicles were prepared at molar ratios between 70:1 and 26:1 (lecithin:triolen) and the microemulsion preparation at a molar ratio of 1:1. The substrate particles were of similar size (220-250 Å) as determined by Bio-Gel A-15m chromatography. Hydrolysis of triolein contained in vesicles or emulsions was similar with respect to pH, temperature, and reaction products. Kinetic studies on vesicles with increasing triolein content showed progressively greater Vmax values (0-0.6 μmol/min/mg), and Vmax for the emulsion was 3.1 μmol/min/mg. Addition of human very low density lipoprotein produced a dose-dependent inhibition with both substrates. The results show that surface-oriented triolein is hydrolyzed in both preparations.

Because of the insolubility of triacylglycerol and cholesterol ester in aqueous systems, the substrate preparations used to assay for acid lipase activity have often been complex and poorly defined containing various detergents, proteins, or emulsifiers. In previous studies we have shown that cholesterol esters or triacylglycerols could be incorporated into unilamellar lecithin vesicles which were stable and effective substrates for an acid lipase from liver and aortic tissue (10, 11). Similar preparations have subsequently been used by other investigators (6, 7, 12, 13).

A lysosomal acid lipase is believed to be responsible for the hydrolysis of triacylglycerols and cholesterol esters contained within lipoprotein particles that enter the lysosomal system by a lysosomal trafficking pathway (14). Because lipoprotein particles have physical properties that would characterize them as microemulsions (15) a model substrate suitable for examining the action of an acid lipase on lipoproteins would be a stable microemulsion. Procedures for preparing stable microemulsions of phospholipid and cholesterol ester have recently been described (16). In this study we report the purification of an acid lipase from rat liver by a modification of the procedure described by Warner et al. (8) and describe the use of triolein-containing phospholipid vesicles and microemulsions as model substrates for this enzyme.

EXPERIMENTAL PROCEDURES

Materials—The following radioisotopes were obtained from New England Nuclear: [1-14C]oleoyl glycerol (99.8 mCi/mmol), dipalmitoyl[3H]lecithin (60 Ci/mmol), [9,10-3H]oleic acid (7.5 Ci/mmol), [phenyl-3H]Triton X-100 (1.58 mCi/mg), bovine serum albumin [methyl-14C] (13.2 μCi/mg), γ-globulin [methyl-14C], (8.7 μCi/mg), and lactoalbumin A [methyl-14C] (21.9 μCi/mg). Sephadex G-150, ConA-Sepharose and blue dextran 2000 were purchased from Pharmacia Fine Chemicals. Bio-Gel A-15m was purchased from Bio-Rad. N-Tetradecyl-N',N'-dimethyl-3-ammonio-1-propane sulfonate (Zwittergent 3-14) was obtained from CalbiochemBehring, and egg yolk phosphatidylcholine (Grade I) was purchased from Lipid Products, Surrey, U.K. Liquisent is a product of National Diagnostics, Sommerville, NJ, and Intralipid (10%) was obtained from Cutter Medical Laboratories, Berkeley, CA. 4-Methylumbelliferyl palmitate and 4-methylumbelliferone were purchased from Sigma. Carbowax PEG-20,000 was purchased from Fisher, and all other chemicals were of the highest reagent grade available. The water used was deionized and distilled in glass to assure purity.

Purification of Rat Liver Acid Lipase—The purification scheme used in this study for rat liver acid lipase was a modification of the procedure of Warner et al. (8) for the isolation of acid lipase from human liver. Purification was monitored using a fluorometric assay (17). Male Sprague-Dawley rats (250-400 g, Charles River Breeding Laboratories, Wilmington, MA) were killed by guillotine. The livers (75 g wet weight) were rapidly excised, placed into ice-cold buffer I (0.25 M sucrose, 0.01 M Tris, pH 7.4, and 1 mM EDTA) and minced. The tissue was homogenized in 4 volumes of buffer I using 3 passes of a Teflon glass homogenizer with a clearance of 0.025 inch, and the procedure was repeated using a pestle allowing only 0.12 inch clearance. Unless otherwise noted, all the following manipulations were

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Acid Lipase Action on Vesicles and Microemulsions

performed at 4 °C. The homogenate was centrifuged at 1800 × g for 10 min and the pellet was discarded. A lysosomal fraction was obtained by centrifuging the supernatant at 14,000 × g for 30 min, and the resulting pellet was resuspended in 160 ml of buffer II (1% Triton X-100, 10 mM sodium acetate, pH 5.0, 1 mM 2-mercaptoethanol, 1 mM MnCl₂, and 1 mM CaCl₂) by homogenizing for 3 min in a Waring Blender. This suspension was then sonicated at 150,000 × g for 30 min in a Beckman Ti 60 rotor and the supernatant used for further purification.

A column of Con A-Sepharose (1.6 × 8 cm) was pre-equilibrated in buffer II. The supernatant was applied at a rate of 1 ml/min. After the solution was passed completely over the column once, the effluent was collected, and the column was cleaned overnight with the aid of peristaltic pump. The column was then washed with 150 ml of buffer III (0.5% Triton X-100, 33% ethylene glycol, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 20 mM sodium acetate, pH 5.0, 1 mM MnCl₂, and 1 mM CaCl₂) followed by an additional 100 ml of buffer IV (0.5% Triton X-100, 33% ethylene glycol, 10 mM 2-mercaptoethanol, and 20 mM sodium acetate, pH 5.0). The column bed containing the absorbed enzyme was then removed from the column and the enzyme eluted batchwise at 25 °C with four 40-ml aliquots of a solution containing all the components of buffer IV plus 0.5 M α-methylmannoside. A total of 0.1 M NaCl, 0.01 M Tris, pH 7.4, and 0.02% sodium azide. All samples were eluted with the equilibration buffer at a flow rate of 10 ml/h. Fractions containing enzymatic activity were pooled and stored at −20 °C.

The combined supernatant was concentrated 10-fold by dialysis against 1.5 liters of 30% Carbowax PEG 20,000 at 4 °C for approximately 50 h. Four-ml aliquots were then applied to a Sephadex G-150 column and pre-equilibrated in buffer IV (buffer IV: 0.1% Triton X-100, and eluted by ascending chromatography with the equilibration buffer at a flow rate of 10 ml/h). Each successive extraction the supernatants were combined and stored at −20 °C.

The Sephadex G-150 column was packed with Sephadex G-150 and eluted with 0.1 M NaCl, 0.01 M Tris, pH 7.4, and 0.02% sodium azide. The designated amounts of labeled and unlabeled triolein so that after resuspension in aqueous buffer the concentration of each lipid was 14.1 mM. These suspensions routinely contained 0.35 μCi/ml of [3H]lecithin and 0.5–2.0 μCi/ml of [14C]triolein.

The resulting suspensions were then sonicated continuously under a nitrogen atmosphere with a Branson W-350 sonicator fitted with a standard 0.5-inch horn at a power setting of approximately 3.5. The temperature within the glass-jacketed sonication cell was maintained between 37 and 42 °C. For vesicle preparation, sonication time was 20–30 min and the microemulsion preparation was sonicated for 1 h. The resulting opalescent mixtures were then centrifuged in a Beckman 50 Ti rotor at 40,000 rpm for 60 min at 5 °C. From the ultracentrifuge tube, the upper 1 ml was removed and 4 ml of the remaining infranatant was then transferred to another tube and centrifuged at 42,000 rpm in a Beckman SW 60 Ti rotor for 16 h at 5 °C. The tubes were then fractionated and aliquots assayed chemically or isotope-atomically for phospholipid or triacylglycerol. For subsequent use as substrates, vesicles were obtained in the lower 1.0-ml fraction and the microemulsion was collected in the upper 1.0-ml fraction of the appropriate centrifuge tube.

To characterize the vesicle or emulsion preparation, 0.2-ml aliquots were applied to a Bio-Gel A-15m column (2.6 × 37 cm) that was pre-equilibrated with a solution containing 0.1 M NaCl, 0.01 M Tris, pH 7.4, and 0.02% sodium azide. The designated amounts of labeled substrate in volumes not exceeding 100 μl, and 10 μl of the purified enzyme preparation, all in a final volume of 250 μl. Twenty-min incubations at 37 °C were routinely performed. After incubation the reaction was terminated by the addition of 3.5 ml of benzenechlorormethanemethanol (1.0:0.5:3.2, v/v/v) containing unlabeled oleic acid (0.1 mM) as carrier. An 0.6-ml aliquot of 0.3 M NaOH was then added, and the resulting suspension was mixed for 15 s followed by centrifugation at 1000 × g for 10 min to separate the phases. The free fatty acid was then determined by counting a 1-ml aliquot of the aqueous phase in a scintillation cocktail in a Packard Tricarb 300 CD programed to calculate the disintegrations per min for each isotope.

To isolate lipoproteins, freshly collected human serum was adjusted to 0.1% EDTA and fractionated by sedimentation or centrifugation in a Beckman 50 Ti rotor. VLDL (d < 1.006 g/ml) and LDL (d = 1.006–1.063 g/ml) fractions were obtained as described previously (23) and dialyzed extensively against 0.15 M NaCl and 1 mM EDTA, pH 7.4, prior to use. As assay for Lipase Activity—Assays using vesicles or microemulsions as substrates were performed similarly. Incubation tubes (13 × 100 mm test tubes) contained 50 mM sodium acetate, pH 4.4, 2.5 mM 2-mercaptoethanol, 0.5 mM EDTA, the designated amount of labeled substrate in volumes not exceeding 100 μl, and 10 μl of the purified enzyme preparation, all in a final volume of 250 μl. Twenty-min incubations at 37 °C were routinely performed. Following incubation the reaction was terminated by the addition of 3.5 ml of benzenechlorormethanemethanol (1.0:0.5:3.2, v/v/v) containing unlabeled oleic acid (0.1 mM) as carrier. An 0.6-ml aliquot of 0.3 M NaOH was then added, and the resulting suspension was mixed for 15 s followed by centrifugation at 1000 × g for 10 min to separate the phases. The free fatty acid was then determined by counting a 1-ml aliquot in 10 ml of a scintillation cocktail in a scintillation counter. Under these conditions 98% of the free fatty acid partitioned into the upper phase. All measurements were made in duplicate.

As assay performed to determine the products of the reaction were scaled to 1 ml. Incubations were terminated by the addition of 20 volumes of chloroform:methanol (2:1, v/v), followed by 5 volumes of 0.5% NaCl and 1 mM H₂SO₄. The lower phase was removed, dried under a stream of nitrogen, and spotted on a thin layer plate (Silica Gel G) in a minimal amount of solvent. The plate was chromatographed in a solvent system of hexane:diethyl ether:acetic acid (70:30:5, v/v/v). The lipids were then scraped off and analyzed for radioactivity with Liquisint as the scintillation cocktail in a Packard Tricarb 300 CD programed to calculate the disintegrations per min for each isotope.

RESULTS

Purification of Acid Lipase—Table I summarizes results from a typical purification procedure. Activity was measured using a fluorometric assay employing surfactant-stabilized emulsions of 4-methylumbelliferone palmitate as substrate exactly as described by Warner et al. (17). Fluorescence was measured using a Turner fluorometer (model 111) with excitation at 360 nm and emission at 450 nm with 4-methylumbelliferone as standard.

The abbreviations used are: VLDL, very low density lipoprotein; LDL, low density lipoprotein.

1
This assay was reliable and reproducible at all purification stages and was effective in the presence of the relatively high concentrations of Triton X-100 and ethylene glycol required during the purification and subsequent storage. The minimum specific activity reported is 2000–3000 nmol/min/mg which is an order of magnitude lower than that reported by Warner et al. (8) for the human liver lipase but was comparable to that reported by other workers for acid lipase obtained from several sources (1, 24). The enzyme was purified at least 80-fold from the lysosomal fraction. This value was not precise due to difficulty in obtaining an accurate protein determination, presumably due to interference by Triton X-100 which gave high blanks and which we were unable to remove by extensive dialysis, surfactant exchange, absorption to polystyrene beads, or incubation with phospholipid vesicles. This problem of obtaining accurate protein values on the Sephadex G-150 eluate was encountered with the Lowry procedure (18), Coomassie blue binding (25), fluorescamine labeling (26), and absorbance techniques. Attempts to further purify the acid lipase by carboxymethylcellulose, Sephadex LH-20, octyl-Sepharose, or Sephacryl S-200 column chromatography produced no appreciable increase in specific activity but losses in total activity were observed.

Fig. 1 shows the elution profile of the enzyme from the Sephadex G-150 column which was calibrated with radiolabeled proteins. The estimated molecular weight was 69,000

<table>
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<th>Step</th>
<th>Volume (ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>Activity (unit/ml)</th>
<th>Specific activity (unit/mg)</th>
<th>Yield (%)</th>
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<td>0.018</td>
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<td>2666</td>
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</table>

Fig. 1. Elution profile of acid lipase activity following gel filtration on Sephadex G-150. Column was pre-equilibrated and eluted using buffer IV but containing 0.1% Triton X-100 (see text). Activity was monitored using 4-methylumbelliferyl palmitate as substrate. The column was standardized using blue dextran 2000, V₀, γ-globulin, 150 kDa; albumin, 69 kDa; lactoalbumin A, 18 kDa; and ¹⁴H₂O, Vₜ.

FIG. 2. Effect of Triton X-100 on the sedimentation of acid lipase in sucrose density gradients. Samples of the purified acid lipase were dialyzed against 10 mM acetate buffer, pH 5.6, containing 0.1% Triton X-100. Aliquots (0.2 ml) were applied onto 10–30% sucrose gradients either lacking or containing 0.1% Triton X-100. Following centrifugation, the activity in each fraction was measured using 4-methylumbelliferyl palmitate as substrate. ■, activity in gradients containing Triton X-100; □, activity in gradients not containing Triton X-100; ●, per cent sucrose. Standards: γ-globulin, 7 S; albumin, 4.6 S.

Fig. 3. SDS-polyacrylamide gel electrophoresis of acid lipase at different stages of purification. Lane A, lysosomal fraction following solubilization with 0.1% Triton X-100. Lane B, fraction following elution from ConA-Sepharose. Lane C, fraction following gel filtration on Sephadex G-150. Lane D, standards: albumin (Mₛ = 68,000); ovalbumin (Mₛ = 43,000); and chymotrypsinogen (Mₛ = 25,000). For lanes A and B material was applied directly to the gel. For lane C the fraction was concentrated approximately 50-fold prior to application. Based on a Kᵥ of 0.42. An independent estimate of molecular weight of the pooled fractions from the Sephadex G-150 column was obtained by sucrose density gradient ultracentrifugation (Fig. 2). When the gradient was run in the absence of Triton X-100, activity was not detected in any fraction even if Triton X-100 was added to the assay tubes subsequent to centrifugation. In the presence of 0.1% Triton X-100, activity was readily detected as a single peak sedimenting as a particle with a sedimentation coefficient of 4.5 S corresponding to a molecular weight of 67,000 Da, based on calibration with ¹³C-labeled albumin and γ-globulin. Fig. 3 shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the fractions from different stages of purification. Following
Acid Lipase Action on Vesicles and Microemulsions

gel filtration, a major band on the gel was observed corresponding to 69,000 Da. A minor band also was observed corresponding to 77,000 Da. Thus, chemical and enzymatic procedures suggest that rat liver acid lipase has a molecular weight between 67,000–70,000 Da and was approximately 85% pure at the final purification step. A minimum molecular weight of 29,000 for the human acid lipase was reported (8) while purified acid lipases from rat liver, human placenta, bovine thyroid, and human leukocyte have been reported ranging from 31,000–74,000 (1, 5, 7, 9, 24).

Preparation and Characterization of Lipid Substrates—The substrates used in this study were prepared by sonicating dispersions of lecithin and triolein of defined molar ratios between 70:1 to 1:1 (phospholipid: triolein), centrifuging the mixtures for 1 h at 150,000 × g to sediment titanium and float large emulsion particles, and then centrifuging at 200,000 × g for 16 h. The resulting preparations were characterized as either vesicles or microemulsions depending on the sedimentation or flotation following 16 h of ultracentrifugation. For all preparations, less than 1% of the total lipid sedimented during the first centrifugation for 1 h. When the initial molar ratio was 70:1, no lipid was detected at the air-water interface of the tube, but as the molar ratios were altered to increase the triolein content, progressively more lipid floated to the surface. At a molar ratio of 16:1, 16% of the triolein and 8% of the phospholipid were localized in a creamy layer at the surface, and for the preparation of molar ratio 1:1, about 96% of the triolein and 12% of the phospholipid floated.

Table II shows the distribution of labeled lipid in the ultracentrifuge tube following prolonged centrifugation (16 h at 200,000 × g) of the infranatants obtained from the 1-h centrifugation of samples containing initial molar ratios of 70:1, 1:1, and 1:1. For the 70:1 preparation (sample 1) over 65% of the total lipid sedimented to the lower part of the tube, and the remaining lipid was distributed throughout the tube. The molar ratio was essentially constant in all fractions. For the 16:1 preparation (sample 2), again most of the lipid sedimented, but the molar ratio of the lower fraction was 27:1 whereas the particles that did not sediment were enriched relative to triolein. About 9% of the triolein associated with a small amount of lecithin floated to the surface suggesting the presence of microemulsions in the mixture. When the 1:1 mixture (sample 3) was centrifuged, about 60% of the lipid added to the tube floated to the surface with a molar ratio of 1:1 in that fraction. The lesser amounts of lipid distributed throughout the tube had molar ratios from 2.5 to 7.3. Chemical analysis for triacylglycerol and phospholipid was also performed on the fractions in the different tubes and the data were consistent with the values obtained based on specific activities of the labeled lipids. The data indicated that under appropriate sonication conditions, starting mixtures of 70:1 to 16:1 molar ratio produce mainly vesicles whereas microemulsions are formed when the starting molar ratio was 1:1.

The particles isolated by prolonged ultracentrifugation were also characterized by gel filtration (Fig. 4). The sedimenting fractions containing molar ratios of 70:1 and 27:1 eluted mainly as a single peak which was included in the column. The 70:1 preparation eluted at a volume corresponding to that of unilamellar lecithin vesicles which are prepared in the absence of triolein, whereas the 27:1 preparation eluted as a broader peak at a volume corresponding to slightly larger particles. The microemulsion prepared by flotation also was included in the column and eluted at a volume similar to that of the 27:1 vesicles. For all preparations, both the [3H]lecithin and [14C]triolein coeluted in the same region, although the molar ratio differed somewhat in the ascending and descending portions of the peaks suggesting slight heterogeneity within each of the preparations. In all cases, small amounts of labeled lipid were noted at the void volume, presumably due to aggregation of the vesicles or microemulsions. A small tritium peak was also found at the total volume, but chemical analysis indicated no phospholipid in this region, and the radioactivity was found to be an impurity in the [3H]lecithin.

Enzymatic Studies—The hydrolysis of triolein incorporated into either phospholipid vesicles (70:1 molar ratio) or microemulsions under different assay conditions is shown in Fig. 5. Although the amount of fatty acid formed was consistently greater when microemulsions were used as substrate, no major differences between the two substrates were seen with respect to the effect of temperature or pH. Optimal activity was observed over a relatively broad temperature range between 37 and 50 °C. The pH optimum for both substrates was about 4.3 and activity was negligible at pH values above 6.5. Hydrolysis increased with incubation time for at least 2 h with both substrates and was linear for about 30 min. The reaction rate was directly proportional to the amount of enzyme from 0–0.4 µg corresponding to 0–20 µl of the purified enzyme preparation. When more enzyme was added, the reaction rate decreased. This behavior was attributed to the presence of Triton X-100 in the enzyme preparation (see below). Based on the data in Fig. 5, assays were routinely performed at pH 4.4 for 20 min at 37 °C, and 0.18 µg (10 µl) of enzyme was added.

Because Triton X-100 and ethylene glycol were present in the enzyme preparation, the effect of these substances on the standard assay was tested. Triton X-100 inhibited hydrolysis of both vesicles (85 µM triolein) and microemulsions (500 µM triolein) in a dose-dependent manner. Fifty per cent inhibition

<table>
<thead>
<tr>
<th>Sample</th>
<th>[3H]Lecithin</th>
<th>[14C]Triolein</th>
<th>Lecithin/triolein</th>
<th>molar ratio</th>
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<td>22.6</td>
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<td>1:1</td>
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</tbody>
</table>
Acid Lipase Action on Vesicles and Microemulsions

was observed at concentrations of about 0.01% and 0.005% Triton X-100 for the vesicle and microemulsion, respectively. Ethylene glycol had no effect when added in amounts 5-fold greater than that normally included in the standard assay. Oleate, a reaction product, was added as the sodium salt and had no appreciable effect at concentrations up to 250 \( \mu \text{M} \)

To determine the reaction products, the reaction mixture was extracted by the procedure of Polch et al. (27) and the distribution of the labeled products determined by thin layer chromatography. When vesicles (70:1 molar ratio) were used as substrates and incubation was for 45 min, 5% of the labeled triolein was hydrolyzed and the molar distribution of fatty acid, mono-, and diglyceride was 3:2:1, respectively. Using the microemulsion as substrate under similar conditions, about 8% was hydrolyzed and the relative distribution of products was 5:3:2. Following prolonged incubation for 6 h at 37 °C, up to 46% of the triolein was hydrolyzed with proportional more mono- than diglyceride. In all cases, the data indicated that monoglyceride was not hydrolyzed in the assay system and all the fatty acid formed was derived from di- or triolein degradation.

Incubations were also performed to determine the stability of the substrates following incubation at standardized conditions. Following the reaction, samples with either the 60:1 or 27:1 vesicles or the 1:1 microemulsion were applied to a Bio-Gel A-15m column and the elution profiles obtained were virtually identical with those shown in Fig. 4.

Phospholipase activity in the enzyme preparation was tested by preparing unilamellar vesicles that contained tritiated lecithin labeled in either the choline or fatty acid moieties. These vesicles were incubated with the enzyme for prolonged time periods up to 2 h under standardized conditions and the reaction mixtures analyzed for water-soluble products using the extraction described for the lipase assay or the procedure of Polch et al. (27). No detectable phospholipase activity was observed using vesicles lacking triolein or using vesicles containing triolein (70:1 molar ratio).

Fig. 4A shows the effect of concentration using vesicles (molar ratios of 60:1, 50:1, and 27:1 and with the microemulsion (molar ratio 1:1) as substrate. Saturation was observed with each of the vesicle substrates but the maximal reaction rate was progressively greater as the vesicles were more enriched in triolein. A sigmoid shape was observed when vesicles were used as substrates (Fig. 6A). When using the microemulsion as substrate saturation was observed with no evidence of a sigmoid-shaped plot. The maximal activity was about 3.1 \( \mu \text{mol/min/mg} \), clearly greater than that obtained for any of the vesicle preparations.

Fig. 6B shows a double reciprocal plot for the microemulsion both in the absence and presence of 10 \( \mu \text{g} \) of Triton X-100, an amount approximately equal to that contained in 10 \( \mu \text{l} \) of the purified enzyme preparation. Without the added detergent, a straight line was obtained giving values of 230 \( \mu \text{m} \) and 3.1 \( \mu \text{mol/min/mg} \) for the apparent \( K_m \) and \( V_{max} \), respectively. When Triton X-100 was added the plot was nonlinear at the lower substrate concentrations. Also shown in Fig. 6B is the data obtained using the larger emulsion particles (molar ratio 1:1:6) that were prepared by flotation from the 1-h centrifugation of the initial sonication mixture. This preparation has the same \( V_{max} \) as the smaller emulsion although the apparent \( K_m \) was greater (1800 \( \mu \text{m} \)).

Double reciprocal plots for the vesicle preparations are shown in Fig. 6C and nonlinearity was observed in all cases. This nonlinearity was even more obvious at substrate concentrations lower than that shown in the figure and became still more pronounced if Triton X-100 were added to the reaction mixture (data not shown). The data in Fig. 6C is consistent with the presence of a substance in the enzyme preparation (Triton X-100) that interacts irreversibly with the substrate, producing nonlinearity when the ratio of detergent to substrate is high. Overestimation of a substrate (triolein) in a reaction would produce sigmoid velocity versus substrate plots and nonlinearity for double reciprocal plots (28, 29). Estimation of the amount of triolein removed from the substrate pool by Triton X-100 may be approximated by linearization of the double reciprocal plots shown following sequential subtraction of small (2 \( \mu \text{m} \)) quantities of substrate from each experiment. When correlation coefficients for each line were
Fig. 5. Characteristics of the assay for acid lipase using either vesicles (molar ratio 70:1) or microemulsions (molar ratio 1:1) as substrate. All incubations were performed at 37 °C for 20 min at pH 4.4 with 0.18 µg of protein unless stated otherwise. A, effect of incubation temperature. Each assay tube contained either 12 µM triolein as vesicles or 688 µM triolein as microemulsions. B, effect of pH. Each assay tube contained either 40 µM triolein as vesicles or 228 µM triolein as microemulsions. C, effect of incubation time. Each assay tube contained either 10 µM triolein as vesicles or 1000 µM triolein as microemulsion. D, effect of enzyme concentration. Each assay tube contained either 24 µM triolein as vesicles or 1000 µM triolein as microemulsions. The enzyme preparation was assumed to contain 18 µg/ml. Each point is the average of duplicate determinations. Similar data were obtained using vesicles of molar ratio 27:1.

Fig. 6. Effect of substrate concentration on acid lipase activity. A, varying amounts of either vesicles or microemulsions were added to assay tubes and the reaction assayed under standardized conditions. □, vesicles with molar ratio 60:1; △, vesicles with molar ratio of 50:1; ○, vesicles with molar ratio of 26:1; ●, microemulsions of molar ratio 1:1. B, double reciprocal plots of kinetic data using the microemulsion as substrate. C, double reciprocal plot of kinetic data using vesicles as substrate.

Fig. 7. Effect of LDL, VLDL, and Intralipid addition on acid lipase activity. A, vesicles as substrate. All assay tubes contained vesicles of molar ratio 60:1 at a final concentration of 28 µM triolein. Increasing amounts of either LDL (○), VLDL (△), or Intralipid (■) were added to the reaction mixture prior to incubation under standardized conditions. Amount of substance added was expressed as the total amount of neutral lipid (cholesteryl ester plus triglyceride) added per assay tube. Control value for vesicle-associated triolein hydrolysis was 0.155 µmol/min/mg. B, microemulsions as substrate. All assay tubes contained microemulsions (molar ratio 1:1) at a final concentration of 640 µM triolein. Control value for microemulsion-associated triolein hydrolysis was 2.18 µmol/min/mg.
optimized, \( V_{\text{max}} \) for each vesicle type did not change significantly but the apparent \( K_m \) values for all vesicles were approximately 30 \( \mu \)M.

The effect of human VLDL and LDL on hydrolysis of both vesicles and microemulsions is shown in Fig. 7. For both substrates LDL was a more effective inhibitor than VLDL when the data was expressed as the amount of neutral lipid (triacylglycerol plus cholesteryl ester) added. 50% inhibition was produced approximately 5 and 100 \( \mu \)g of neutral lipid from LDL and VLDL, respectively. Inhibition also was produced by Intralipid, a commercially available triacylglycerol emulsion.

**DISCUSSION**

Several lipases have been shown to hydrolyze neutral lipid contained in surface monolayers (30, 31). These data strongly suggest that lipolysis occurs on the surface of biological emulsions. Lipoprotein particles, which have the properties of microemulsions (15), are degraded intralysosomally, presumably by several enzymes including the acid lipase. To assess the mechanism by which the acid lipase degrades microemulsions such as lipoproteins, we prepared model substrates of either microemulsions or vesicles containing neutral lipid. Sonication of mixtures of phospholipids and cholesteryl esters have been shown to generate vesicles or microemulsions depending on the starting lipid ratio (16). Using a similar approach, we prepared vesicles and microemulsions containing lecithin and triolein.

The maximum solubility of surface-oriented triolein in a vesicle was reported to be about 3–4 mol % based on NMR studies (32). Thus, the vesicles we prepared at 4 mol % would have essentially all the triolein accessible to the surface of the particle and also contain a saturated surface of triolein. When the triolein content is increased to a molar ratio of 1:1, microemulsions are formed in preference to vesicles. Emulsions of lecithin and triolein, are reported to have a surface containing 2–5 mol % triolein (33). In order to compare the hydrolysis of vesicle- and microemulsion-contained triolein, great care was taken to characterize the substrate preparations with respect to both size and composition. Theoretically the vesicle containing 4 mol % triolein and the microemulsion should have nearly identical surface compositions. Since the sizes of vesicle and microemulsion preparations are very similar, it is, therefore, possible to compare the hydrolysis on a per particle basis.

Comparisons between the hydrolysis of triolein contained in either the microemulsion or the vesicles showed similarities with respect to pH activity profiles and incubation temperature, suggesting that the same enzyme acts on both substrates. The most striking difference between the two types of substrates related to the kinetic data obtained. The \( V_{\text{max}} \) value obtained using the microemulsion or 1:1.6 emulsion was considerably greater than that obtained for any vesicle preparation. Assuming that vesicles with 4 mol % triolein and the emulsions both have saturated surfaces with respect to triolein, the different \( V_{\text{max}} \) values for both substrate types suggest that the concentration of triolein on the surface was not the sole limiting factor in determining the reaction rate.

The difference between the \( K_m \) values observed between the 1:1 microemulsion and the 1:1.6 emulsion again suggests that surface-oriented triolein is hydrolyzed and the greater \( K_m \) for the 1:1.6 emulsion most likely reflects the relatively large amount of triolein contained in the core of these particles and, therefore, not directly accessible to the enzyme. Using the microemulsion as substrate, an apparent \( K_m \) of about 250 \( \mu \)M was calculated based on the total triolein concentration. Assuming that the enzyme acts solely at the lipid-water interface and that approximately 4% of the triolein in the microemulsion is localized on the interface, a \( K_m \) value corrected for surface-oriented triolein of 10 \( \mu \)M is obtained. Presumably triolein in vesicles is distributed randomly throughout the bilayer; thus 67% of the triolein in each preparation is situated in the outer leaflet at any time (34). Therefore, the apparent \( K_m \) calculated for the different vesicle preparations (30 \( \mu \)M) when corrected to account for triolein in the outer leaflet is about 20 \( \mu \)M. When corrected for surface-oriented substrate, apparent \( K_m \) values between vesicles and microemulsions differ by a factor of 2. This difference, although based on several approximations, may reflect different affinities of the enzyme for either particle surface.

The action of lipases on an interface has been reviewed recently by Verger (35). A proposed mechanism for hydrolysis involves binding of the lipase to the surface of a particle, followed by localization of the substrate to the active site. Upon hydrolysis of one substrate molecule the enzyme is free to either dissociate from the particle or continue to hydrolyze substrate on the same particle. Should dissociation be less likely, the diffusion of substrate within the particle to the active site may become important in determining the maximal velocity. The greater \( V_{\text{max}} \) in microemulsions compared to vesicles could be due to a more rapid movement of core triolein to the surface in microemulsions than the lateral movement of triolein in the vesicles.

When comparisons were made between different vesicle preparations the \( V_{\text{max}} \) increased proportionally as the relative amount of triolein in the vesicle increased. This is consistent with an increasing concentration of substrate available on the surface of the particle, which would lessen the lateral diffusion time between enzyme and substrate, possibly explaining the increasing \( V_{\text{max}} \).

Criticism concerning the use of emulsions for studying the properties of a lipase were based on size heterogeneity and the lack of detailed compositional data for previously used emulsion preparations (35, 36). The microemulsions used in this study have not been previously described and were characterized with respect to size and composition. Using such preparations it was possible to show that surface-oriented triolein was hydrolyzed by the acid lipase and that the core of an emulsion can effect the rate of neutral lipid hydrolysis. Such microemulsions are amenable to more detailed studies on the physical properties of surface and core phases using other techniques. The effectiveness of the microemulsion as a substrate for acid lipase, the similarities to lipoproteins with respect to size, and the demonstrated ability of lipoproteins to inhibit lipase activity toward the microemulsion suggest that these synthetically prepared particles may be useful in elucidating the mechanism by which acid lipase acts on naturally occurring microemulsions such as lipoproteins.

**REFERENCES**

Acid Lipase Action on Vesicles and Microemulsions

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