Photometric Microdetermination of Serum Lipase with a Phenyl Laurate Substrate

Abraham Saifer and Guta Perle

The Raderecht and Moskau procedure for pancreatic lipase (6) has been modified for determining the small amounts of this enzyme present in normal blood serum. The present investigation confirmed their findings: that phenyl laurate provides a stable easily-prepared substrate of uniform composition; that the substrate is specifically split by serum and pancreatic lipase; and that the reaction product (phenol) can be readily determined by a simple and sensitive colorimetric procedure.

The lipase units are expressed in the same manner as are phosphatase units; i.e., milligrams of phenol liberated per 100 ml. serum at 37° in 30 min. The normal range, based on 40 sera, was found to be 2.5–5.6 units.

The serum lipase values were shown to be significantly elevated in a number of cases with acute pancreatic disease. The advantage of this procedure over others presently employed for diagnostic evaluation of these conditions is discussed.

In a review article dealing with the fat-splitting enzymes in blood, Overbeek (1) concluded that: "The situation with regard to knowledge of these enzymes is thus one of confusion and uncertainty. It is clear that development of standard methods of determination of the various enzymes is very much needed." This statement agrees with Lagerlöf's view (2) that the diagnostic importance of serum levels of pancreatic lipase has been doubtful for some time because of the problems in its determination.

According to the classification scheme of Richter and Croft (3), the serum lipases are classified with esterases, which hydrolyze fats,

From the Biochemistry Department, Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn 3, N. Y.

This investigation has been aided by Grant B-285-C6 from the U.S. Public Health Service.

The authors wish to acknowledge the invaluable aid of Dr. Walton H. Marsh, biochemist, and Mr. Ben Fingerhut, senior chemist, Kings County Hospital, Brooklyn, N. Y., in supplying many of the samples with elevated amylase values used in this study. Mrs. Lillian Salowitz' editing and typing of the manuscript is also gratefully acknowledged.

Received for publication Aug. 29, 1960.
olive oil, and long-chain fatty-acid esters of alcohols, phenols, and naphthols. Many other types of esterases are also able to split short-chain fatty-acid esters with monohydroxyalcohols, and long-chain fatty-acid esters with glycerin. However, the speed of hydrolysis of these substrates will vary with the enzyme used, the pH, the buffer composition, and the cumulative effects of the activators and inhibitors of the system (4). Katz (5) has stated that to be practical and reliable a method for determining the minute amounts of normal pancreatic serum lipase should utilize small amounts of serum, short incubation periods, colorimetric readings, and a suitable substrate, thus excluding the overlapping effects of other enzymes. The recently published serum lipase method of Raderecht and Moskau (6), using buffered phenyl laurate as the substrate and cholate as an activator, appeared to meet all the requirements for the routine clinical determination of this enzyme. These authors, however, had applied the procedure to serum enriched with pancreatic tissue lipase. In attempting to apply this method to normal serum, it was found that the difference in readings between the unknown and control tubes were too small, and the errors too large, to yield significant results. These authors (6) did not give a range of normal values for serum lipase and did not adequately define their enzymatic units, so that the method could not be reproduced in other laboratories.

The purpose of this investigation was to adapt the Raderecht-and-Moskau photometric procedure (6) to routine serum lipase analyses, to utilize it for determining the range of a normal population in units based on a standard phenol unit, and to test its applicability for the diagnosis of acute pancreatic disease (7).

Experimental

Reagents

*Phenyl laurate* (Eastman Kodak No. 7885). Store in the freezing compartment of a refrigerator. For substrate solution, dissolve 250 mg. of phenyl laurate in 25 ml. of acetone. Keep under refrigeration.

*Barbital-acetate buffer*. Dissolve 9.714 gm. sodium acetate (3 H₂O) and 14.714 gm. sodium barbital in 500 ml. of CO₂-free water. Add 500 ml. 0.1N HCl and 1300 ml. CO₂-free water. The pH of buffer (7.4) should be checked with a pH meter.

*Sodium cholate, 20.0%.* Dissolve 20 gm. in 100 ml. of distilled water.

*Obtainable from Mann Research Labs., New York, N. Y.*
Folin-Ciocalteu's phenol reagent. Dilute 1:2 for use (one part reagent plus one part water).

Sodium carbonate, 15.0%. Dissolve 15 gm. of anhydrous sodium carbonate in 100 ml. of distilled water.

Phenol standard. Stock solution (1 mg./ml.): Dissolve 1.00 gm. of pure crystalline phenol in 1 L. of 0.1N HCl. Keep under refrigeration. This solution can be standardized by the addition of an excess of a standard iodine solution and back-titration with standard sodium thiosulfate to a starch end-point. Dilute Standard (0.05 mg./ml.): Dilute 5.0 ml. of stock standard with water to 100 ml. in a volumetric flask. If kept under refrigeration, solution is stable for 1 month.

Substrate Mixture. Prepare fresh for each run by adding slowly with a volumetric pipet with the tip submerged 10 ml. % phenyl laurate to 10 ml. barbital acetate buffer of pH 7.4 and 35 ml. distilled water. Mix by swirling while adding in order to obtain a colloidal suspension.

Procedure

Unknown

Into a 10 × 120-mm. test tube put 0.2 ml. serum or plasma, 5.0 ml. phenyl laurate substrate mixture, and 0.5 ml. sodium cholate solution. Mix and incubate in a 37° water bath for 30 min. Add 3 ml. of dilute phenol reagent and mix. Let stand 10 min. at room temperature and centrifuge tubes at 2000 rpm for 10 min. To 5.0 ml. of the clear supernatant add 2.5 ml. of 15% Na₂CO₃. Mix and let stand 20 min. at room temperature. If a precipitate forms, centrifuge tubes for the last 3 mins. of the incubation period. Read in Klett-Summerson photoelectric colorimeter with a No. 69 filter (or at 690 mμ in a spectrophotometer) against a reagent blank set at zero absorbancy.

Serum Blank

Into a (10 × 120-mm.) test tube put 5.0 ml. of substrate mixture and 0.5 ml. of sodium cholate solution. Mix and incubate at 37° for 30 min. Add 0.2 ml. serum or plasma, followed immediately by 3.0 ml. of diluted phenol reagent. Mix contents. The remaining steps are the same as for the unknown.

Standard

Into a 10 × 120-mm. test tube, put 1.0 ml. of diluted phenol stand-

†Obtainable from Hartman-Leddon Co., Phila., Penna.
ard, 4.7 ml. of water, and 3.0 ml. of diluted phenol (Folin-Ciocalteu) reagent and mix contents. The remaining steps are the same as for the unknown.

Reagent Blank

Into a 10 × 120-mm. test tube put 5.7 ml. of water and 3.0 ml. of diluted phenol reagent. Mix contents. The remaining steps are the same as for the unknown.

Calculations

\[
\text{Lipase (units)} = \frac{\text{O.D. unknown} - \text{O.D. serum blank}}{\text{O.D. standard}} \times 25
\]

where O.D. represents absorbancy. Lipase units are defined in the same manner as phosphatase units; i.e., the amount of enzyme that will liberate 1 mg. of phenol from the phenyl laurate in 100 ml. serum in 30 min. at 37°.

Results

Normal Sera

Forty fasting blood serum samples were obtained from hospital personnel having no signs of illness. Both sexes were about equally represented in this group and ranged in age from about 20 to 50 years. Either the enzyme determinations were performed on these sera within 30 minutes after clotting at 4° or the sera were frozen at −20°. Test runs showed the serum lipase to be stable for at least one week in the frozen state.

A statistical analysis of the serum lipase results, as performed with the method described above, gave a mean value and standard deviation of 3.57 ± 1.09 units, a variance of 1.18 units and a coefficient of variation of 30.5 per cent. The normal range derived from these statistics is 2.5–5.6 units.

Disease Sera

The stoichiometry of the procedure for the pancreatic type of lipase was determined by analyzing increasing aliquots of a 0.1% solution of steapsin (Merck) dissolved in buffer. The results obtained are given in Table 1 (Samples 1–3) and show that the color obtained with the reactions follows Beer’s Law up to 30 lipase units.

The remainder of the data presented in Table 1 are from patients with suspected pancreatic disease, most of whom showed elevated amylase values (above 200 units) (8) just prior to, or simultaneously
Table 1. Lipase and Amylase Values Obtained with Sera from Suspected Cases of Acute Pancreatitis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lipase units</th>
<th>Lipase units plus eserine*</th>
<th>Amylase units ($)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.9</td>
<td>11.3</td>
<td>..</td>
<td>0.1% steapsin, 0.10 ml.</td>
</tr>
<tr>
<td>2</td>
<td>19.1</td>
<td>21.2</td>
<td>..</td>
<td>0.1% steapsin, 0.20 ml.</td>
</tr>
<tr>
<td>3</td>
<td>30.6</td>
<td>30.7</td>
<td>..</td>
<td>0.1% steapsin, 0.30 ml.</td>
</tr>
<tr>
<td>4</td>
<td>6.3</td>
<td>5.6</td>
<td>..</td>
<td>Serum</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>5.5</td>
<td>..</td>
<td>Serum</td>
</tr>
<tr>
<td>6</td>
<td>7.6</td>
<td>7.3</td>
<td>..</td>
<td>Serum</td>
</tr>
<tr>
<td>7</td>
<td>6.7</td>
<td>6.2</td>
<td>..</td>
<td>Serum</td>
</tr>
<tr>
<td>8</td>
<td>7.8</td>
<td>7.8</td>
<td>..</td>
<td>Serum</td>
</tr>
<tr>
<td>9</td>
<td>7.7</td>
<td>..</td>
<td>1000</td>
<td>Serum</td>
</tr>
<tr>
<td>10</td>
<td>2.3</td>
<td>..</td>
<td>646</td>
<td>Serum</td>
</tr>
<tr>
<td>11</td>
<td>7.0</td>
<td>..</td>
<td>828</td>
<td>Serum; diagnosis of acute pancreatitis confirmed at autopsy.</td>
</tr>
<tr>
<td>12</td>
<td>1.1</td>
<td>..</td>
<td>816</td>
<td>Serum; 0.2 lipase units with Cherry-Crandall method (9).</td>
</tr>
<tr>
<td>13</td>
<td>15.6</td>
<td>..</td>
<td>567</td>
<td>Serum; acute pancreatitis.</td>
</tr>
<tr>
<td>14</td>
<td>5.8</td>
<td>..</td>
<td>720</td>
<td>Serum; cirrhosis of liver.</td>
</tr>
<tr>
<td>15</td>
<td>7.2</td>
<td>..</td>
<td>1234</td>
<td>Same patient as 14. Serum taken 1 week later.</td>
</tr>
</tbody>
</table>

*Same procedure as described in text except that before incubating unknowns 0.30 ml. of eserine (3.0 × 10⁻⁴M) was added to each tube. Blanks received 0.30 ml. eserine after addition of phenol reagent and serum.

with the increased lipase values. In Sample 12, which showed a low lipase value (1.1 units) and a markedly elevated amylase value (816 units), a lipase determination performed with the olive oil substrate technic of Cherry and Crandall (9) yielded a low normal value—i.e., 0.2 units.

To rule out the possibility that part of the hydrolysis of the phenyl laurate is due to serum pseudocholinesterase, aliquots of eserine salicylate were added to some of the serum samples (Sample 4-8) in order to inhibit the activity of this enzyme. Since the lipase values obtained with these sera in the presence of eserine were the same as in its absence, it was concluded that pseudocholinesterase has no appreciable effect on the substrate under the experimental conditions employed. Agreement with the results of Katz (5), that eserine has no effect on the action of pancreatic lipase, is shown by the results obtained (Samples No. 1-3) on its addition to steapsin.

The serum lipase results obtained for acute pancreatic disease (Table 1) show that elevated values are obtained in proved cases (Sample 11). That the degree of elevation does not always corre-
spond to that of the amylase values is shown by the results obtained for Samples no. 9 and 10. Samples 14 and 15 are from the same patient with cirrhosis of the liver, taken at different times. The rise in both lipase and amylase values reflect increasing secondary pancreatic involvement with the progression of the disease.

Discussion

There are four types of esterases capable of acting on long-chain fatty-acid ester substrates present in serum: (1) clearing-factor (or lipoprotein) lipase; (2) pseudocholinesterases; (3) liver esterases; and (4) pancreatic lipases. Clearing-factor lipase is not normally active in the blood (10) and, as shown by the results obtained with eserine in this paper, pseudocholinesterase has no overlapping effect under the chosen experimental conditions. The addition of sodium cholate to the system serves both to activate lipase and to inhibit esterases (5, 6). The use of barbital buffer (4) and a pH of 7.4 (6) also serves to make the method specific for lipase and to reduce that of other kinds of esterases.

It has been stated by some investigators (4, 11) that only long-chain fatty-acid esters of glycerol; e.g., olive oil, should be used as a substrate for lipase. Yet, even the best of published methods using this substrate have not been widely utilized in clinical laboratories because the different samples of olive oil vary markedly in their rates of hydrolysis (4), the incubation times are long, and the titrations required are laborious. It is also questionable whether the rate of hydrolysis for triglycerides is the same as that for the di- and monoglycerides that form rapidly as the reaction proceeds. The results obtained in this paper confirm those of Raderecht and Moskau (6) that phenyl laurate provides a stable, easily prepared substrate of uniform composition, which is specifically split by serum and pancreatic lipase and whose reaction product (phenol) can be readily determined by a simple and sensitive colorimetric procedure.

The question of fundamental importance in any proposed method for serum lipase in the clinical laboratory is whether it can readily detect increased amounts of this enzyme in acute pancreatic disease. The results obtained for this enzyme on patients with acute pancreatic disease (Table 1) confirm those previously obtained by Katz (5) using α-naphthyl laurate as a substrate. He reported increases in lipase values up to 10 times the normal in acute diseases of the pancreas, but low results in chronic pancreatic disease. Because of
the difficulty in obtaining adequate numbers of patients with proved acute pancreatitis, the data listed here, while suggestive of the possible utility of the procedure, are insufficient for a more definitive statement. According to Tietz et al. (4) the serum lipase tends to be increased and stay increased in pancreatitis as compared to amylase, and, therefore, the determination of lipase serves to reflect the clinical picture more clearly than that of amylase.

References