On the Determination of "Pancreatitis Lipase" in Serum

Richard J. Henry, Charles Sobel, and Sam Berkman

IN SPITE OF A RATHER VOLUMINOUS LITERATURE on lipolytic enzymes over the past 50 years there appears to be a widespread misunderstanding about the determination of "lipase" in serum as a diagnostic test for acute pancreatitis.

The use of olive oil as a substrate for measuring lipolytic activity can be traced back into the nineteenth century. The extent of hydrolysis was determined by titration of the released fatty acids. In 1911 Rona and Michaelis (1) used tributyrin as substrate, determining the degree of hydrolysis by stalagmometric measurements; i.e. the number of drops from a capillary tube per unit time, which is increased by the decrease in surface tension resulting from hydrolysis. Willstätter and co-workers in 1923 used both tributyrin (2) and olive oil (3) in their studies on pancreatic extracts.

Cherry and Crandall in 1932 (4) applied the olive-oil procedure to serum and found that, following pancreatic duct ligation in dogs, "lipase" increased if olive oil was used as substrate but not when either ethyl butyrate or tributyrin was used. Comfort and Osterberg (5, 6, 7) using the olive-oil technic of Cherry and Crandall found increased values in 83 of 84 cases of pancreatitis. That there is increased serum lipase activity in this disease, when this technic is used, has been confirmed by others (8, 9). Increased values may also occur in carcinoma of the pancreas (10) and hepatic disease (6, 10).

The hydrolysis of tributyrin by serum has been shown to be due to a pseudocholinesterase (9, 11, 12). Goldstein and Roe (13) reported that in cats poisoned by chloroform, primarily a liver poison, there was decreased serum amylase and olive oil "lipase" but increased hydrolysis of ethyl butyrate and tributyrin.
The terminology which is more or less accepted today defines esterases as enzymes which split organic esters of low molecular weight, and lipases as enzymes which split glycerol esters of fatty acids of high molecular weight, i.e., fats. This division is relative only. Esterases have generally been regarded as originating mainly in the liver, while lipases come primarily from the pancreas. Many papers have been published on studies of the kinetics of the lipolytic activity in pancreatic extracts and there is no question that such extracts do split tributyrin as well as many other lower fatty-acid esters. Several European investigators, using stalagmometric measurements with tributyrin as substrate [refs. cited in (14)], have claimed that pancreatic lipase in serum does hydrolyze tributyrin when calcium oleate is present. Lagerlöf (15) reported increased values in 17 of 18 cases of acute pancreatitis by such a method.

By 1948 the validity of the olive-oil method as a diagnostic test for pancreatitis was well established. Because it involved certain technical difficulties laboratories were receptive to a new test that could be run in a shorter period of time than 24 hours. In 1948 Goldstein, Epstein, and Roe (16) modified a method using tributyrin previously introduced by them in 1943 (17). This method is still used in many laboratories despite the lack of any clinical evidence of its validity as a diagnostic test. In fact it was published as an approved procedure for lipase in *Standard Methods of Clinical Chemistry* (18). Subsequent to this publication it was reported (9) that no increase in “tributyrinase” was detected in 8 cases of acute pancreatitis.

The work presented herein was undertaken at the suggestion of the Editorial Committee of *Standard Methods* of the American Association of Clinical Chemists with the view of replacing the tributyrinase method appearing in *Standard Methods of Clinical Chemistry, Vol. 1*, with a method which would be a valid diagnostic test in acute pancreatitis.

**EXPERIMENTAL**

**Substrates**

A brief search was made in the hope that a substrate could be found which could be easily prepared, relatively stable and, above all, would be hydrolyzed more rapidly than olive oil, so that a technic could be evolved requiring a shorter period of incubation. The lipolytic activity of pancreatin (Armour Laboratories, Chicago, Lot No. M 30801) and sera from cases of acute pancreatitis were compared on olive oil by the method of Cherry and Crandall (4), on tributyrin by the method of Goldstein, Epstein, and Roe (16), on glycerol monostearate, and on evaporated milk.
In the cases of milk and glycerol monostearate the Cherry and Crandall technic was followed, substituting milk or a 1% emulsion of glycerol monostearate for the olive-oil emulsion. The milk was brought to pH 7.0 before use. The emulsion of glycerol monostearate was prepared by adding the ester (cosmetic grade, Process Chemical Co., Los Angeles) to boiling water slowly with stirring. Myverol® (Type 18-05, control no. S 11789, Distillation Products Industries, Rochester, N. Y.), a purer sample of the ester, was also used, although emulsification was much more difficult. Typical results, confirmed repeatedly, are shown in Table 1. It is seen that pancreatin hydrolyzed olive oil, the fat in milk, glycerol monostearate, and tributyrin. The lipolytic enzyme in the sera from patients with acute pancreatitis did not hydrolyze milk fat or glycerol monostearate. There was some hydrolysis of tributyrin, but no more than that observed with normal sera. (Normal limits given by Goldstein et al. (16) are 81 to 246 units.) Because of the importance of this observation, sera from 14 cases of acute pancreatitis were studied by the method of Goldstein et al. and by the modified olive oil method of Cherry and Crandall as described later in this communication. The results are shown in Table 2. In not one case was there an elevation of “tributyrinase.” This confirms the report of Bunch and Emerson (9). Actually, the levels are frequently lower than normal.

These experiments indicated a very high specificity of the enzyme or enzymes in sera from cases of acute pancreatitis for triglycerides, and no further search was made for an alternative substrate.

Study of the Olive Oil Method

As a starting point, the technic of Cherry and Crandall (4) was followed.

Titration Curves and Indicators

A sample of oleic acid (Lot no. 288, “purity greater than 99%” Mann Research Laboratories, Inc., N. Y. 6) was weighed and dissolved in ethanol to give a concentration of 0.0658N, assuming it to be 100% pure.

<table>
<thead>
<tr>
<th>Table 1. The Activity of Pancreatin and “Pancreatitis Lipase” as Measured by Various Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive</td>
</tr>
<tr>
<td>oil</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Pancreatin, 1% solution</td>
</tr>
<tr>
<td>Serum from acute pancreatitis</td>
</tr>
</tbody>
</table>
Table 2. **COMPARATIVE RESULTS OBTAINED WITH SERA FROM PATIENTS WITH ACUTE PANCREATITIS BY THE OLIVE-OIL METHOD AND THE METHOD OF GOLDSTEIN ET AL.**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Units olive oil</th>
<th>Units tributyrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>2.9</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>3.2</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>7.7</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>7.8</td>
<td>130</td>
</tr>
<tr>
<td>7</td>
<td>4.4</td>
<td>95</td>
</tr>
<tr>
<td>8</td>
<td>6.0</td>
<td>184</td>
</tr>
<tr>
<td>9</td>
<td>8.8</td>
<td>48</td>
</tr>
<tr>
<td>10</td>
<td>7.3</td>
<td>140</td>
</tr>
<tr>
<td>11</td>
<td>16.3</td>
<td>116</td>
</tr>
<tr>
<td>12</td>
<td>4.3</td>
<td>84</td>
</tr>
<tr>
<td>13</td>
<td>10.1</td>
<td>28</td>
</tr>
<tr>
<td>14</td>
<td>5.8</td>
<td>100</td>
</tr>
</tbody>
</table>

*Normal ranges: up to 1.5 for olive oil and 81 to 240 for tributyrin*

Titration of this solution with 0.0503N aqueous NaOH, using either thymolphthalein or thymol blue as indicator, indicated it to be 0.0651N, or 99% of theoretical. Three milliliters of the oleic acid solution were thus equivalent to 3.88 ml of the standard alkali employed. To obtain a titration curve of the oleic acid in the environment existing in the Cherry-Crandall technic, the first step taken was the titration of a mixture of 3.00 ml of the ethanolic solution of oleic acid, 6.0 ml water, and 0.50 ml M/15 phosphate buffer pH 7.0. Due to the very limited solubility of oleic acid in water, most of the alkali required for titration of the oleic acid was added before dilution with buffer and water and the remaining oleic acid was titrated potentiometrically. Fig. 1A shows this titration curve, as well as the titration curve of a blank composed of a mixture of 6.0 ml water, 0.50 ml buffer, and 3.0 ml ethanol, and the difference between the two curves which is indicated by the broken line. When 3.88 ml alkali had been added, the amount required to react stoichiometrically with the oleic acid, the pH was 10.5.

The experiment was repeated, substituting a mixture of 3.0 ml water, 2.0 ml olive-oil emulsion, and 1.0 ml serum for the 6.0 ml of water. The titration curves for blank, oleic acid, and their difference are shown in Fig. 1B. Above a pH of about 9 the protein is being titrated. The protein, however, is present in equal amount in both blank and the mixture of blank and oleic acid and the stoichiometric end point for titration of the oleic acid still occurs at pH 10.5.
Fig. 1. Titration curves of oleic acid. Model G Beckman pH meter used. A, without serum; B, with serum. Curve 1 = blank; Curve 2 = blank + oleic acid; Curve 3 = difference between Curves 1 and 2.

From the inspection of Fig. 1 A it would appear from extrapolation that the pK of oleic acid in a water-ethanol mixture is about 8. Fig. 1 B indicates that the addition of serum and olive oil emulsion raises the pK to about 8.4. Titration curves of the acid produced from olive-oil emulsion by action of sera from patients with acute pancreatitis are quite similar to that of Fig. 1 B with estimated pK's of about 8.2 to 8.4.

Titration to the equivalence point, pH 10.5, by use of a pH meter, would be the most accurate technic, but also the most laborious. If phenolphthalein is used as indicator, as in the original Cherry-Crandall procedure, only about 70 per cent of the fatty acids released are titrated since the mixture turns pink at pH 8.8. Thymolphthalein, the indicator used by Willstätter and coworkers (3), gives a very pale blue at pH 10.1, becomes distinctly blue at pH 10.5, and a deep blue at pH 10.8. The end-point with thymolphthalein is not very sharp, but it is estimated that if
one titrates to a "distinct" blue, the error is less than 5 per cent of the total titration. This is considered adequate for routine determinations.

The purpose of the addition of an organic solvent such as alcohol or an alcohol-ether mixture prior to titration has been: (1) to stop enzymatic activity, and (2) to put the free fatty acids into solution for titration. If titration is not delayed the first object is of no consequence. As for the second purpose, identical results were obtained with and without the addition of the alcohol, although the pK of the fatty acids appeared to be somewhat lower in the presence of the alcohol.

Blanks

The blank titration includes the alkali required by the buffer, emulsion, and serum to raise the pH to the end point used. Serum inactivated at 70° for 5 minutes and added to the blank tube at the beginning of the incubation period has been proposed in some textbooks [e.g. (19)]. It was found safer and more convenient to follow one of two alternative procedures: (1) add the serum to the incubated blank immediately before titration, or (2) add 1.0 ml. serum to 3.0 ml. water and 0.5 ml. buffer, inactivate in boiling water for 5 to 10 minutes, cool, add olive-oil emulsion, and incubate.

It was found in several hundred experiments that the blank titration for any particular olive-oil emulsion remained practically constant. This confirms the claim of Cherry and Crandall. The purpose of running a blank, therefore, is to check on technic and reagents, and one should be run with each set of determinations. It is not necessary to run a blank with each serum and, in fact, if one unknown is run and the quantity of sample is limited, a blank employing another serum can be used.

Substrate Emulsion

In the original Cherry-Crandall technic, equal amounts of olive oil and 5 per cent gum acacia in water containing 0.2% sodium benzoate were emulsified. In a search for a more stable emulsion, emulsification procedures using Carbapol 934 (20), bile salts (21), and polyvinyl alcohol (22) have been proposed. Cherry-Crandall mixtures, emulsified either by passing through a hand homogenizer or in a Waring Blender for 15 minutes, were compared with the method of preparation employing Carbapol 934. Both of the Cherry-Crandall emulsions were stable for 2 weeks in the refrigerator and then a separation into two phases occurred. The Carbapol mixture was stable for less than one week and was unusable after separation into two phases occurred. Originally, results with these
three emulsions were essentially the same, but upon breakdown, the Carbopol emulsion gave significantly lower results. On the other hand, the Cherry-Crandall emulsions were usable for at least two more weeks after separation when shaken before use. Possibly this may be due to the fact that the Carbopol 934 emulsion separated into a top oil phase and a bottom oil-in-water emulsion, whereas the Cherry-Crandall emulsion separated into a top oil-in-water emulsion and a bottom water phase.

Throughout this work, Old Monk Olive Oil (Old Monk Co., N. Y.) was used. Although identical results were obtained with U. S. P. Olive Oil, the Old Monk product was found easier to emulsify, requiring a maximum of five passages through a hand homogenizer to produce a smooth, white emulsion.

**Time of Incubation**

Figure 2 gives the degree of hydrolysis as a function of the time of incubation for 5 sera. The original Cherry-Crandall technic called for a 24-hour incubation period. The shape of the curve relating hydrolysis and time deviates sufficiently from zero order so that there is little increase in hydrolysis after 16 hours. Practically, it may be more convenient
for the laboratory to set up, late in the afternoon, all samples received during the day and titrate them the next morning. A 4-hour incubation time has been suggested (9). In the case of the 5 samples shown in Fig. 2, the values at 4 hours would average 50 per cent (range of 44 to 58 per cent) of those obtained at 16 hours. Specimens received in the morning thus could be reported out the same day, but the advantage would be lost for specimens received in late afternoon. Use of two different periods of incubation may lead to confusion.

**Temperature of Incubation**

Figure 3 gives the results obtained with two pancreatitis sera at different temperatures. The optimal temperature is at about 40°. It would be more convenient for most laboratories, however, to incubate at 37°.

**Buffers and pH**

No extensive study of the effect of varying the pH and the buffer was made on the lipolytic action of sera from acute pancreatitis. The initial pH of a mixture of 3.0 ml. water, 2.0 ml. emulsion, 0.5 ml. M/15 phosphate buffer (pH 7.0), and 1.0 ml. serum was 7.2. After incubation, the final pH's, without the addition of alcohol, of mixtures using sera con-

![Graph](image-url)

**Fig. 3.** Effect of temperature on rate of hydrolysis. Time of incubation 6 hours. Solid circles, 6.5 units at maximum. Open circles, 3.2 units at maximum.
taining 5 and 10 units of lipase were 6.8 and 6.6, respectively. Substitution of the M/15 phosphate buffer by M/3 phosphate buffer pH 7.0, the strength used by Comfort and Osterberg (5), by 0.4 M NH₄OH—NH₄Cl buffer pH 8.4, and by 0.2 M trishydroxymethylaminomethane (THMAM) buffer pH 8.4 (23) produced the results shown in Table 3. With the latter two buffers the reaction mixture was adjusted to pH 8.4 prior to incubation. The strength of phosphate buffer did not appear to influence results, nor did the use of either of the other two buffers at pH 8.4.

**Enzyme Concentration**

In Fig. 4 the degree of hydrolysis (units) is plotted against the volume of serum used in the test. The concentrations of substrate and buffer were kept constant. The relationship is not linear.

**Activators**

As determined by studies on pancreatic juice or extract, calcium ions (3) and bile salts (3, 24) activate "lipase." It is believed that the fatty acids released during hydrolysis inhibit enzyme activity, and calcium ions act by removal of the fatty acids through formation of unionized or insoluble soaps. A solution of oxgall in glycerine was prepared by dissolving 5 Gm. oxgall (Bacto) in 5 ml. water, adding 50 ml. glycerine and heating in boiling water. Figure 4 shows the effect of adding 1 ml. of this bile solution to the reaction mixture. It would appear that there was a slight activation. To determine the effect of calcium ions, the technic proposed by Nothman and co-workers (25) was compared with the technic employing M/15 phosphate buffer. The Nothman reaction mixture contains 3.0 ml. water, 5.0 ml. 2% calcium acetate, 5.0 ml. 0.5% sodium barbital, and 1.0 ml. serum. Results obtained with this mixture (Table 4)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phosphate pH 7.0</th>
<th>NH₄OH—NH₄Cl pH 8.4</th>
<th>THMAM pH 8.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M/15</td>
<td>M/3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.3</td>
<td>.</td>
<td>4.7</td>
</tr>
<tr>
<td>2</td>
<td>3.4</td>
<td>3.3</td>
<td>.</td>
</tr>
<tr>
<td>3</td>
<td>1.4</td>
<td>1.4</td>
<td>.</td>
</tr>
<tr>
<td>4</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>5</td>
<td>2.2</td>
<td>1.9</td>
<td>.</td>
</tr>
<tr>
<td>6</td>
<td>3.3</td>
<td>3.2</td>
<td>2.6</td>
</tr>
<tr>
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<td>2.0</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.2</td>
</tr>
</tbody>
</table>
were considerably lower than those obtained with phosphate buffer. When the calcium acetate was omitted and water substituted the results agreed quite well with those obtained with phosphate. The addition of 2.5 ml. 2% calcium chloride to a reaction mixture in which the phosphate buffer was replaced by THMAM buffer, pH 8.4, also resulted in considerable inhibition. It is concluded, therefore, that calcium ions, at least in the concentrations employed, inhibit rather than activate the lipase present in sera from patients with acute pancreatitis.

**Effect of Hemolysis**

It has been reported (20) that hemoglobin is an inhibitor of “pancreatitis lipase.” This was confirmed in a series of experiments in which it was found that although a serum hemoglobin concentration of 0.16% produced no inhibition, at about 0.5% there was about 50 per cent inhibition and at 2.5% there was almost complete inhibition.

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**Figure 4.** Effect of enzyme concentration (5 different sera).

**Table 4. The Effect of Bile and Calcium on Results Obtained by the Olive-Oil Method**

<table>
<thead>
<tr>
<th>Sample</th>
<th>M/15 phosphate</th>
<th>M/15 phosphate + 1 ml. bile</th>
<th>Nolhman with calcium acetate</th>
<th>Nolhman without calcium acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.8</td>
<td>13.9</td>
<td>6.1</td>
<td>12.0</td>
</tr>
<tr>
<td>2</td>
<td>4.6</td>
<td>4.9</td>
<td>0.9</td>
<td>5.0</td>
</tr>
<tr>
<td>3</td>
<td>3.8</td>
<td>4.5</td>
<td>1.3</td>
<td>4.3</td>
</tr>
<tr>
<td>4</td>
<td>1.4</td>
<td>1.3</td>
<td>.</td>
<td>.</td>
</tr>
</tbody>
</table>

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Reproducibility

As determined from 8 replicates on each of two sera containing 3.5 and 5.5 units of activity, the precision (95 per cent limits) was ±10 per cent.

Stability of Samples

Six sera, ranging in activity from 4 to 10 units, were followed at room temperature for one week and no change in activity was observed. One of these was followed for an additional week during which time it showed heavy bacterial growth and an increased lipolytic activity, presumably due to bacterial action. Sera stored at refrigerator temperature were followed for 3 weeks and showed no change in activity.

Normal Values

The 95 per cent limits of the normal range as determined on 24 adults by the “normal equivalent deviate” method (26) after log transformation of the data, were as follows: 0.06 to 0.89 unit for a 4-hour incubation period and 0.2 to 1.5 units for a 16 to 24-hour incubation period. Since the lower limits differ from 0 units by less than the experimental error of the method, the normal ranges can be taken as “up to 0.9 unit” and “up to 1.5 units,” respectively.

Bunch and Emerson (9), using essentially the same technic as employed here for the 4-hour technic, obtained limits of 0.06 to 0.87 units. Their limits for a 24-hour technic were 0.1 to 1.0, but they used the calcium-barbital buffer system of Nothman (25) for this series. Comfort and Osterberg (7) considered the top normal to be 1.3 units for the Cherry-Crandall technic.

DISCUSSION

The data presented clearly reveal the striking specificity of the lipolytic enzyme or enzymes occurring in sera from patients with acute pancreatitis. Since pancreatitis lipase differs considerably from lipase in pancreatic extracts with reference to substrate specificity and activators, it is evident that caution should be taken in applying data obtained from work on pancreatic lipase to the problem of the determination of lipase in pancreatitis serum. To avoid confusion in terminology, the term “pancreatitis lipase” is suggested, recognizing the possibility that the pancreas may not be the sole source of the enzyme since elevated values by the olive-oil method have been reported in liver disease (6, 7).

Whether or not the enzyme present in normal serum is the same as that
appearing in pancreatitis serum is not certain. There seems to be no question but that normal sera do have weak activity in hydrolyzing olive oil. Others (27) have agreed with this conclusion.

This study has ended up more or less where it started, namely, with the Cherry-Crandall technic, using an olive-oil emulsion as substrate. The only modification made has been the titration of the liberated fatty acids to pH 10.5 either by use of a pH meter or by use of thymolphthalein as indicator.

Seligman and Nachlas (28) have proposed a method employing \( \beta \)-naphthyl laurate as substrate, and they reported obtaining increased values in acute pancreatitis. Since this has been questioned (29), and relatively little data are as yet available (30), this method must as yet be considered to be in the investigative stage.

**SUMMARY**

The specificity of the lipolytic activity occurring in sera from patients with acute pancreatitis for olive oil has been demonstrated. The "lipase" method proposed by Goldstein and co-workers, employing tributyrin as substrate, does not measure "pancreatitis lipase." "Pancreatitis lipase" differs from the enzymes present in pancreatic extracts so that, if the former is the subject of concern in experimental studies, sera from patients with pancreatitis must be used as the source of the enzyme.

Several variables involved in the use of olive oil as substrate have been examined, and the technic finally arrived at is essentially that proposed by Cherry and Crandall with the exception of the use of thymolphthalein as indicator in the titration. Normal values for this technic were determined.

**REFERENCES**