A Convenient and Efficient Method for the Determination of the Digestibility of Fats With Pancreatic or Other Lipases

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A LIPASE splits a fat into the fatty acid and glycerol. If the amount of free fatty acid produced is determined by titrating with KOH solution, a measure of the amount of hydrolysis due to the fat-splitting enzyme is obtained. Since fats do not mix with water, they are digested in the form of an emulsion. The amount and speed of digestion depend largely upon the following factors:

(1) The nature of the fat
(2) The lipolytic enzyme used
(3) The character of the emulsion
(4) The temperature
(5) The reverse reaction as caused by the products of hydrolysis.

The present method suggests notable improvements for the production of a better emulsion which can be reproduced with such uniformity that curves can be plotted for the entire digestion period. The products of the hydrolysis are constantly neutralized as formed, resulting in a smooth digestion of greater efficiency.

The work as carried out by Weinstein and Wynne, 1936,[7] Davis, 1936,[2] Balls, Matlack and Tucker, 1937,[1] Hartwell, 1938,[3] and Kelsey, 1939,[4] will aptly illustrate the conventional methods as used up to the present time. All sought to insure suitable emulsification by means of gum or soap, activators, and mechanical agitation. The amount of hydrolysis was determined by titrating with alcoholic potassium hydroxide.

These methods involved the removal of aliquot portions, or the preparation of individual samples for each determination. The enzyme action was killed by the addition of alcohol. In some instances the precipitate was removed from the sample by filtration. The pH was either not considered at all or controlled by methods which did not permit accurate determinations. This separate handling of samples, and the transference from one vessel to another by means of pipettes caused considerable variation.

The object of the present investigation was to find a better and easier method for carrying out hydrolysis experiments with pancreatic lipase; and by using this method to make a comparative study of the digestibility of some of the commercial edible fats. A comparative study of the activity of two commercial lipases was also made, but since they differed but little in activity, only one need be included in this paper.

Attempts were made to follow the methods suggested, but several difficulties presented themselves. It seemed advisable, therefore, to maintain a constant pH by adding aqueous KOH solution directly to the digestion mixture to which five drops of phenolphthalein had been added. Since phenolphthalein has a color change at pH 8.3, it was possible to keep the digestion mixture at approximately the same alkalinity, which is also within the range at which pancreatic lipase is most active. Thus two procedures were reduced to one—pH control and titration results were accomplished by one operation. Furthermore, it eliminated the laborious removal of samples and the filtrations.

It was found necessary to have a uniform emulsion. This could only be produced by having the same agitation for each sample. Figure 1 shows how five samples can be run at one time at 40°C in a controlled water bath. The line shaft and 45 degree bevel gears insure that each digestion-mixture receives the same amount of stirring.

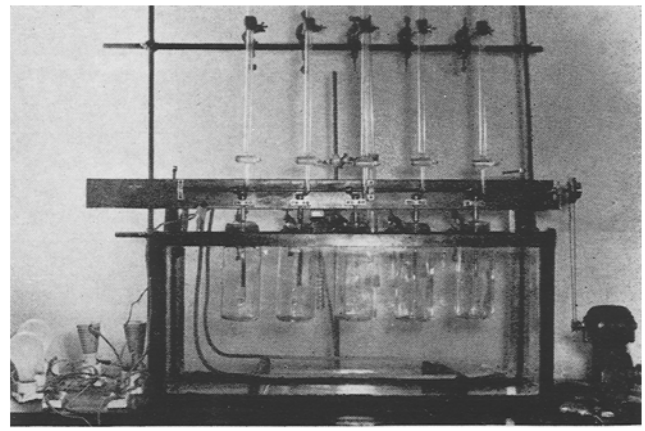


Figure 1.

The use of good emulsifying agents, constant temperature, uniform stirring, and continual neutralization to a pH of 8.3 resulted in uniform reproducible digestions. Previous workers have reported the amount of digestion after a given length of time when the percentage of hydrolysis has become constant. Obviously the rate of digestion up to this point of maximum digestion is of considerable interest. For example, a change in the direction of the digestion curve might well illustrate the presence of two separate enzymes, the action of the one following that of the other.

Graph 1 shows a typical digestion curve together with its check. It will be noted that the points fall well into line and that the progress of the digestion is easily followed. A blank run was always made, without fat present, so that the small amount of KOH used up by reagents could be deducted in the conventional manner.

Gum ghatti was used as the emulsifying agent because much less gum was needed than with gum acacia which had been used by many research workers. It was found that this gum had a much greater emulsifying power and that 1 gram of gum was capable of emulsifying 20 grams of fat when no electrolyte was added. Emulsification with gum ghatti had several advantages —much less gum was needed, no lumping occurred in the preparation, and emulsions were formed with much less difficulty. From the standpoint of emulsification, it was found most satisfactory of the emulsifying agents tried; and from the standpoint of hydrolysis it was found to compare favorably with gum acacia.

Determinations must be carried out as uniformly as possible and the description of a typical run will illustrate the procedure.

STOCK SOLUTIONS:

(1) Fat emulsion with gum ghatti
Moisten 4.8 grams of gum ghatti with 16 ml of distilled water. Add 24 grams of fat with stirring (electric mixer) on hot water bath. Stir until smooth. Add hot water to make exactly 300 ml Keep stirrer running until a smooth milky white emulsion is obtained. (About ten minutes.)
(2) Calcium chloride, 20 grams per liter.
(3) Sodium oleate, 20 grams per liter.
(4) Sodium glycocholate, 1 gram per 50 ml (20 grams per liter).
(5) Albumin, 2 grams per 50 ml (40 grams per liter).
(6) Lipase Powder, 0.5 grams per 25 ml. Prepare shortly before use. (20 grams per liter.)

ADDITION OF STOCK SOLUTIONS: (See apparatus figure 1)

Stock solutions were all previously attemperated to 40°C. Fifty ml of the fat emulsion (containing 4 grams fat) were put into each of the five bottles placed in the water bath at 40°C. Ten ml of each of the 4 activator solutions were added.

The substrate emulsion was stirred for about 45 minutes to insure complete uniformity and 10 ml of lipase solution put into each of the bottles. The 50 ml of fat emulsion, 4 — 10 ml portions of activator and 10 ml of enzyme solution make exactly 100 ml in each bottle.

This 100 ml contains: 4.0 grams of fat
0.8 gram of gum ghatti
0.2 gram of calcium chloride
0.2 gram of sodium oleate
0.2 gram of sodium glycocholate
0.4 gram of albumin
0.2 gram of lipase powder

The burettes above each bottle allow for the constant addition of N/10 KOH solution so as to keep the pH at 8.3. Phenolphthalein was used as an indicator and the pH checked at suitable intervals. The more active the lipase the more KOH solution will be used up to any specified time.

TABLE 1.

Sample Report Sheet for Hydrolysis of Lard
(Saponification Number 193)

Total Time	Trial A ml KOH	Trial B ml KOH	Blank ml KOH	Percent Hydrolysis A	Percent Hydrolysis B
Blank	1.2	1.0	1.1	.....	.....
15 min.	37.2	36.9	1.5	26.0	25.8
30 min.	64.4	63.3	1.7	45.5	44.8
45 min.	76.9	75.8	1.9	54.5	53.8
60 min.	80.7	79.4	2.0	57.2	56.2
90 min.	83.9	82.8	2.5	59.2	58.5
2 hrs.	86.6	84.3	3.1	60.8	59.1
3 hrs.	88.1	85.7	4.3	61.0	59.4
4 hrs.	89.5	88.3	4.7	61.5	60.5

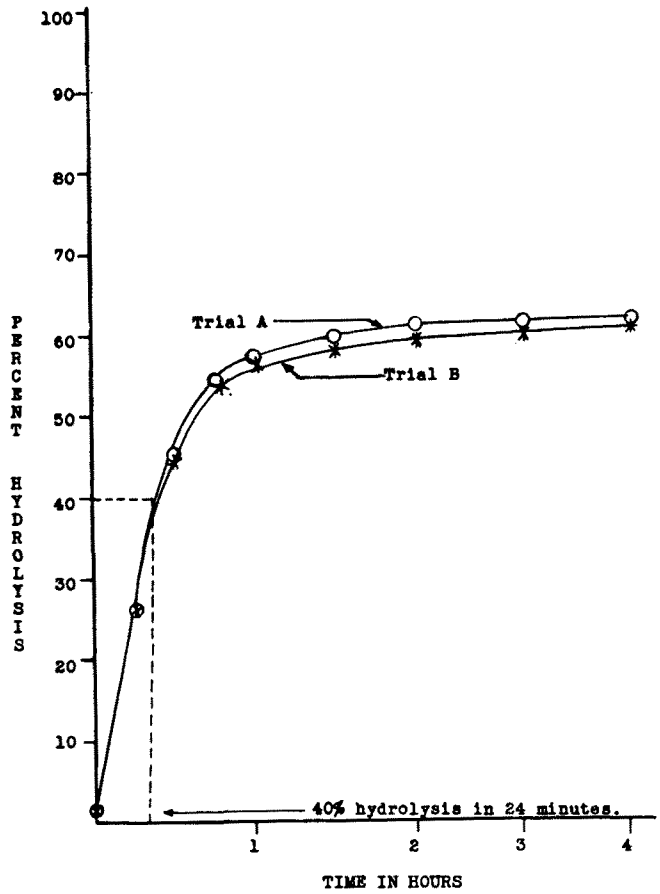
In order to compare the activity of various lipase samples on a given substrate, it is suggested that the time required for 40% digestion be noted. This was used by Longenecker and Haley[5] to determine the lipase units for comparing various samples of lipase. Activity figures are calculated according to the formula as devised by Northrup and Hussey,[6] namely, $a = 1/hg$ where a = activity figure

h = time in hours for 40% digestion
g = grams of enzyme used.

The per cent hydrolysis as shown here is determined by the mg of KOH used up to a specified time as compared to the mg of KOH used for the conventional saponification number.

Graph 1

Hydrolysis of Lard with Pancreatic Lipase



It may well be argued that it would be better to determine the saponification value by using the fat emulsion containing everything except the enzyme. If this is done one may find lipases that will give as high as 150% hydrolysis. In other words, the lipase can do more fat splitting than boiling alcoholic KOH solution over a period of two hours. Since hydrolysis figures of over 100% are reported in the literature, it is interesting to note the wide variation in so called "saponification values" when a substrate emulsion is boiled with alcoholic KOH. (Table II.)

TABLE II

"Saponification Numbers" for Lard and Various Emulsions As Used

Lard only. Conventional saponification number	193.0
Lard, gum ghatti (no water)	204.0
0.2 gm gum ghatti, 12.5 ml water	6.7
Lard, 12.5 ml water with no agitation	12.2
Lard, 0.2 gm gum, 12.5 ml water with no agitation	31.4
Lard, 0.2 gm gum, 12.5 ml water with some agitation	74.0
Lard, 0.2 gm gum, 12.5 ml water with more agitation	96.5
Substrate emulsion* Trial 1	91.5
Substrate emulsion* Trial 2	98.0
Substrate emulsion* Trial 3	126.0

(*The sample contained 1 g. lard, 12.5 ml water, 0.2 g. gum ghatti; it was emulsified as prepared for hydrolysis and was saponified with alcoholic KOH as in preceding determinations.)

It is readily seen from the above table that water prevents the full action of the KOH on the fat. Hot alcohol is enough of a fat solvent to complete the action in about two hours but hot *dilute* alcohol will at best allow the saponification to go to only 50% completion in two hours. The influence of agitation in making for more saponification is clearly evident from the table above.

This latitude in calculation is further illustrated be-

low. A commercial hydrogenated fat showed a saponification value of 182 by the conventional method but only 91.5 when determined on the fat emulsion. The percentage of hydrolysis would then be:

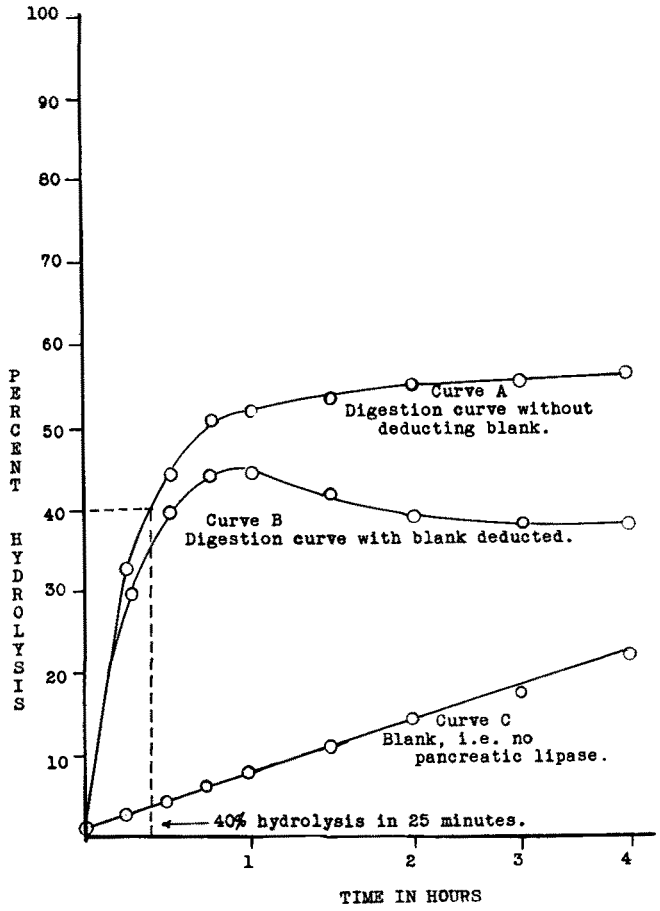
Time	15 min.	1 hr.	2 hrs.	4 hrs.
Per cent of conventional saponification number	26.2	57.5	61.0	61.7
Per cent of emulsion saponification number	53.8	118.2	126.1	127.8

The hydrolyis of a sample of oleomargarine was especially interesting because it showed the presence of fat splitting ingredients within the oleomargarine itself. The sample consisted of two portions:

A—A vegetable fat.
B—A liquid claimed to contain a specified number of U.S.P. units of Vitamins A and D.

Graph 2

Hydrolysis of Margarine with Pancreatic Lipase

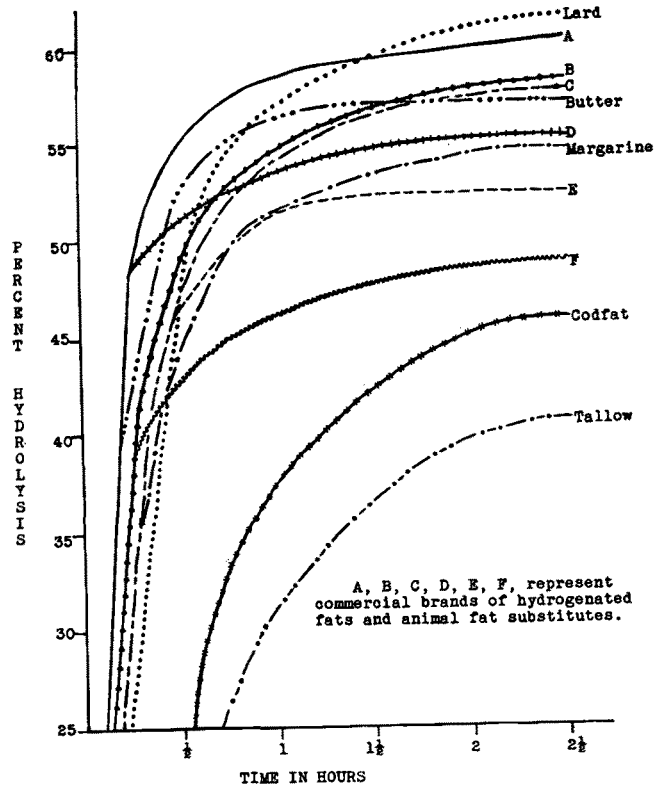


Since the blank has no enzyme added, no hydrolysis would be expected. The graph above shows that 20% hydrolysis took place, even though no lipase was added. It is logical to conclude that the portion B, added for its vitamin content, also contained a fat splitting enzyme.

In conclusion, a chart showing the digestibility of several commercial fat products by the same pancreatic lipase is shown. It will be seen that most of these fats are broken down to the extent of 50% within one-half hour. The codfat and tallow are not sold as edible fats.

Graph 3

Summary of Hydrolysis of the Fats



Summary

This method for carrying out hydrolysis experiments with pancreatic lipase is convenient and easy to operate; it allows five determinations to be carried out at one time, thus making it possible to make comparisons since the same conditions exist at the given time; it shows a smooth course of reaction in every case; it gives more complete hydrolysis since the acids are used up as formed; it keeps the pH on the alkaline side under constant control and in the range where the enzyme is most active; it eliminates the removal of aliquots and the killing of the enzyme; it makes it possible to make determinations directly in the digestion mixture.

A fat emulsion cannot be completely saponified by alcoholic KOH solution in any reasonable length of time. Such a saponification number is usually about half of the conventional value. This is due to the water present in such a fat emulsion.

A sample of oleomargarine was found to contain a fat-splitting enzyme.

Digestion curves for several commercial fats have been presented.

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