Influence of bile salt, pH, and time on the action of pancreatic lipase; physiological implications

BENGT BORGSTRÖM
Department of Physiological Chemistry, University of Lund, Lund, Sweden

SUMMARY The difference in relative rates of hydrolysis of the 1,3- and the 2-ester bonds of triglycerides (50-100:1) catalyzed by pancreatic lipase results in a rapid net hydrolysis of the 1,3-ester bonds at an enzyme level that effects only limited hydrolysis of the 2-ester bond. The net hydrolysis of the 1,3- or primary ester bonds proceeds until a steady state has been established between rate of hydrolysis and synthesis of the primary ester bonds.

This equilibrium state involving the primary hydroxy groups is strongly dependent on the pH of the incubation medium and the presence of bile salt and is reached starting either from triglyceride or from fatty acid and alcohol (using the 2-mono-glyceride analogue 2-octadecenyl-glycerol ether). At equilibrium at pH 5.0 about 80% of the primary hydroxy groups are acylated. This unexpected finding seems to be explained by the presence of a two-phase (oil/water) system presenting an interphase where the enzyme is acting at a very low water concentration and allowing lipid-soluble reaction products to be removed into the oil phase.

Bile salts affect pancreatic lipolysis under slightly acid conditions by changing the equilibrium state more towards hydrolysis, i.e., accumulation of monoglyceride. Other detergents so far tested inhibit pancreatic lipase.

The physiological implication of these results seems to be that the transformations catalyzed by pancreatic lipase in the presence of bile salt form a system for the continuous supply of monoglycerides and fatty acids to the brush border of the intestinal cells.

It has been well documented (1, 2) that pancreatic lipase preferentially catalyzes the hydrolysis of the 1- and 3-ester bonds of triglycerides. One would therefore expect the consecutive formation, during hydrolysis, of 1,2-diglyceride and 2-monoglyceride, and the accumulation of 2-monoglyceride has indeed been demonstrated under certain conditions (3, 4).

Pancreatic lipase hydrolysis of triglycerides is further complicated by the exchange of the fatty acids of the primary ester bonds with the fatty acids formed during lipolysis (5). The formation of new ester bonds to be inferred from such an exchange reaction has also been shown to result in the acylation of 1,2-diglycerides to triglycerides (5).

Because of the irreversibility of the splitting of the last ester bond, that of the 2-monoglyceride, the hydrolysis of triglycerides catalyzed by pancreatic lipase can be brought to completion in vitro if the product of enzyme and time of incubation is large enough (5, 6).

Pancreatic lipase exerts its physiological function in the lumen of the upper small intestine under rather specific conditions, i.e., in the presence of bile and at a slightly acid pH (7). Very few studies so far have been undertaken to define the course of pancreatic lipolysis under these conditions. It has been reported, however, that bile salts shift the pH optimum of pancreatic lipase from pH 8–9 in their absence to the slightly acid values prevailing in upper small intestine (8).

The present studies have been undertaken to evaluate the effects of bile constituents and pH on the complex reactions catalyzed by pancreatic lipase.

MATERIALS AND METHODS

Enzyme
As source of pancreatic lipase lyophilized pancreatic juice from rats and humans was used. Lyophilized pancreatic juice contains no esterases and electrophoresis shows only one zone with lipase activity (9). The lipase content of 1 ml of rat pancreatic juice corresponds to approximately 600 lipase units determined according to Marchis-Mouren et al. (10). The lyophilized human
pancreatic juice (11) contained approximately 10 lipase units per milligram of protein. In a series of experiments, intestinal content collected from upper jejunum of human beings during the digestion of a test meal was employed (7).

**Substrates**

Triolein was used as substrate. Unlabeled triolein was a gift from Karlskhamns Oljefabrik, Sweden.

Glycerol-C14 trioleate and glycerol-HP trioleate were prepared from labeled glycerol and the acyl chloride according to standard procedures and purified on silicic columns. The radiopurity of both compounds as revealed by thin-layer chromatography (TLC) was at least 98%.

2-(Δ9-Octadecenyl-9,10-HP)-glycerol ether was synthesized from the sodium alcoholate of 1,3-benzylidene glycerol and Δ9-octadecenyl-9,10-HP-p-toluen sulfate essentially as described by Gupta and Kummerow (12). The compounds were purified by silicic acid chromatography and the radioactivity found by TLC to be better than 98%.

1,3-Di-O-dodecyl-glycerol 2-(oleate-1-C14) was synthesized by acylating the 1,3-diether (made from 1,3-dichloro-hydrin and sodium dodecyl alcoholate) with 1-C14-oleoyl chloride. The product was purified by silicic acid chromatography.

2-(Oleoyl-1-C14)-oxy-malonic acid 1,3-octadecenyl diester was synthesized from hydroxymalonic acid. This was acylated with 1-C14-labeled oleoyl chloride to give 2-oleoyl-oxy-malonic acid, which was then treated with oxalyl chloride. The acid chloride obtained was added to octadecenyl alcohol. The substance was purified by silicic acid chromatography and the purity checked by TLC.

Sodium taurodeoxycholate (NaTDC) and sodium glycocholate (NaGC) were synthesized and purified as described by Hofmann (13) and were better than 97% pure. Lauryl sulfate was a commercial product that was used without further purification. Oleoyl taurine was synthesized using the same procedures as those used for synthesis of the taurine-conjugated bile acids. The solutions were made up to 0.15 M with respect to sodium ions. Lyssolecithin was obtained from egg lecithin using snake venom phospholipase A essentially according to Hanahan’s procedure (14). It was crystallized from chloroform and ethanol.

**Buffers**

In the pH range 5.0–8.0, 0.15 M sodium phosphate buffers were used; at pH 8.6 a borate buffer was used.

**Incubations**

The substrate, in heptane, was transferred to 10-ml glass ampoules, the heptane was evaporated off and buffer, detergent if used, and lipase were added to a final volume of 4 ml. The ampoules were closed and shaken (140 strokes/min, amplitude 50 mm) at 37° for the specified periods. In the experiments in which human intestinal content was used, 4 ml of the latter was added to the ampoules and the sample was treated as described in the text of the tables. Extraction of lipids was performed as described earlier (15).

**Radioactivity Assay**

In the experiments in which labeled substrates were employed, the total lipids after extraction were dissolved in 2 ml of heptane. Aliquots (50 μl) were taken for determination of total activity and the same amounts put on Silica Gel G thin-layer plates. The plates were developed with a mixture of acetic acid, methanol, ethyl ether, and hexane 1:1.5:10:45 (16) and sprayed with iodine in methanol, and the mono-, di-, and triglyceride spots from the plate was scraped off and transferred to the counting vessels. The radioactivity was determined by means of an automatic Tri-Carb Scintillation spectrometer using dioxane with PPO and POPOP as scintillators (16).

The mean recovery of activity in the tri-, di-, and monoglyceride spots from the plates in 11 experiments using glycerol-C14 trioleate as substrate was 92.4% of the radioactivity applied to the plate.

Free glycerol-C14 was determined by counting 1 ml of the lower phase (obtained after extraction of the lipids) in 10 ml of dioxane. In the experiments with human intestinal content, the lower phase was colored and effected a marked quenching. To 5 ml of the lower phase of these samples was added 1 ml of 10% BaCl2 solution, 2 ml of 0.3 N NaOH, an Agla micrometer, and bromothymol blue as indicator.

After filtration a clear colorless solution was obtained which was used for assay of radioactivity in free glycerol as above.

**Determination of Fatty Acids**

Fatty acids were determined by titration of aliquots of the total lipid extract using 0.02 N NaOH, an Agla micrometer, and bromothymol blue as indicator.

Fatty acids liberated were also calculated in the glycerol-labeled trioleate experiments from the sum of 3 × free glycerol + 2 × monoglyceride + 1 × diglyceride.

**RESULTS AND DISCUSSION**

In the experiments described in the present investigation the triolein used as substrate was not, or was at best poorly, emulsified at the beginning of the experiments. Thus, the interphase to start with was small but well defined, and any changes that occurred during the ex-
In Fig. 1 is shown the composition of the glyceride mixture obtained when triolein is hydrolyzed in phosphate buffer pH 8.0 for 1 hr in the presence of increasing amounts of pancreatic lipase. The amount of free glycerol formed is represented on a molar basis in a cumulative fashion. The amount of free fatty acids formed is given as a percentage of the total amount of fatty acid present at the beginning as triglyceride fatty acids. The amount of free fatty acids increases up to a level of 50% and then additional enzyme causes a relatively smaller increase in fatty acids. Parallel to the change in fatty acid liberation there is a disappearance of triglyceride and the appearance of mono- and diglycerides. At high enzyme concentration the monoglycerides are the dominant glyceride species present in the incubation mixture. Free glycerol production is largely proportional to the enzyme concentration. Free glycerol production was determined by titration with KOH. The data in Fig. 2 show that during the conditions of these experiments the net conversion of diglyceride to monoglyceride is equal to the net conversion of monoglyceride to glycerol. Similarly the formation of diglyceride from triglyceride equals the conversion of diglyceride to monoglyceride. The last step in the reaction, the hydrolysis of the mono-
glyceride, is again not saturated with respect to enzyme even at the highest levels used.

Figure 3 shows a detailed study of the effect of pH and of bile salt on the final composition of the glycerides obtained when triolein is hydrolyzed with rat pancreatic lipase for 1 hr in the presence of a high enzyme concentration, i.e. one for which the addition of enzyme does not greatly affect the relative composition of the glyceride mixture (Fig. 2). It can be seen that in the absence of bile salt a profound change in the glyceride composition is obtained when the pH of the incubation medium is changed from 5.8 to 7.4. The relative proportion of monoglycerides increases and becomes the dominating glyceride fraction at pH 7.0 and above. At low pH values the triglyceride is the dominating glyceride species. The most marked effect of bile salt is an increase in the monoglyceride content under acid conditions of the expense of triglyceride and an increase of triglyceride at the expense of monoglyceride at higher pH values.

When interpreting the above data we recalled the results of previous experiments showing that during hydrolysis of triglycerides by pancreatic lipase the fatty acids in the 1- and 3-positions of the glyceride equilibrate with the free fatty acids of the incubation medium (5). It then appeared that the glyceride mixtures obtained at high enzyme concentrations represented equilibrium states between hydrolysis and synthesis for the reactions:

\[ \text{triglyceride} + \text{H}_2\text{O} \rightleftharpoons 1,2\text{-diglyceride} + \text{fatty acid} \]
\[ 1,2\text{-diglyceride} + \text{H}_2\text{O} \rightleftharpoons 2\text{-monoglyceride} + \text{fatty acid}. \]

The lipase-catalyzed exchange reaction involving the primary ester-groups of the glyceride molecule referred to above has so far only been observed to occur simultaneously with a net decrease of ester bonds and has been considered not to represent any net synthesis of ester bonds (20). If, however, it reflects the existence of an equilibrium state between synthesis and hydrolysis the same end products should be reached starting either from the ester or from the 2-monoglyceride and fatty acids. Because of the ready isomerization and hydrolytic

![Graph showing product formation during lipolysis at different pH values.](image-url)

**Fig. 3.** Products formed during lipolysis at different pH values in the presence and absence of bile salt. Substrate 32 \( \mu \text{Eq} \) of glyceryl-C\(^\text{18} \) trioleate, rat pancreatic juice protein added corresponding to 0.08 ml of the original juice. Final volume 4 ml, incubated 1 hr at 37\(^\circ\).
splitting of 2-monoglycerides by lipase, the stable analogous 2-(Δ₉-octadecenyl-9,10-H³)-glycerol ether was used in these experiments. Incubation of the glycerol ether with oleic acid, molar proportions 1 to 2, in the presence of pancreatic lipase in buffer solution resulted in a net formation of primary glyceride ester bonds, the equilibria obtained being dependent on the pH of the incubation mixture (Fig. 4). In these experiments (in which human pancreatic lipase was used) a much longer incubation time was used with the glycerol ether, 24 hr compared to 1 hr in the triolein experiment, to ensure that steady states were reached. Prolonged incubation (up to 96 hr) did not significantly change the results, indicating that steady states had been reached for hydrolysis and synthesis of the primary ester bonds.

As can be seen in Fig. 4 the molar percentages of mono-, di-, and triglycerides and of the corresponding glycerol ethers present at equilibrium, starting either from triglyceride or from the 2-alkenyl-glycerol ether and oleic acid, are strikingly similar.

At pH 5.0 and in the absence of bile salts approximately 80% of the primary hydroxy groups of the glycerol ether are acylated when the molar ratio of alcohol groups to acid is 1:1; at pH 8.6, on the other hand, only about 10% are acylated. The steady state under slightly
Fig. 8. Effect of different bile salts on the composition of the glyceride mixtures during pancreatic lipolysis at pH 5.8. Lipase level corresponding to 0.1 ml of rat pancreatic juice in a total volume of 4 ml. Incubation at 37° for 1 hr. NaTDC, sodium taurodeoxycholate, NaGC, sodium glycocholate, NaDC, sodium deoxycholate, NaC, sodium cholate, NaL-S, sodium lauryl sulfate, NaO-T, sodium oleoyl taurine.

The equilibrium state is also dependent on the concentration of bile salt in the incubation medium. Increase in bile salt (pH 5.8) up to 24 μEq/ml increases the proportion of monoglyceride and decreases that of di- and triglycerides (Fig. 6). There is, however, no difference if the same amount of bile salt in the incubation medium is used at constant volume or at constant concentration (Fig. 7).

The most marked effect of bile salt on the steady state composition of the glyceride mixture is the relative increase in monoglyceride at slightly acid pH values. The effect of some different bile acids at pH 5.8 is seen in Fig. 8. It is obvious that different bile salts, conjugated or nonconjugated, generally have the same effect; they increase the proportion of monoglyceride. Differences exist among different bile salts that are not well understood at the present time. As has been observed earlier sodium lauryl sulfate (31) completely inhibits pancreatic lipolysis of triglycerides, as does also oleoyl taurine. It is of interest that both of these detergents, at low concentration, stimulate the hydrolysis of micellar monolein (15).
It is known that bile salt forms mixed micelles with monoglycerides and fatty acids and that the solubility of tri- and diglycerides in these micelles is very low (18). The effect of added bile salt at low pH values is the same as an increase in pH, in the absence of bile salt. This probably is due to an effect of the bile salt on the ionization of the fatty acids, in line with the demonstration by Schmidt-Nielsen of an increased solubilization and ionization of fatty acids in detergent solutions (19). The ionization might also shift the fatty acids from the oil phase to the water phase where they are no longer available for the enzyme, which probably acts in the oil/water interphase (17). The bile salt effect on the equilibrium state of the primary ester bonds at acid pH values therefore appears to be related to interactions between bile salt on the one hand and substrate and split products on the other, rather than to any direct effect of bile on the enzyme.

The effect of pH and of bile salt on the equilibrium state thus would be analogous, both these factors influencing the availability of the reaction products at the site of the enzyme action. A migration of one of the products of the reaction (the ionized fatty acid) from the site of action of the enzyme (= the oil phase (17)) could be predicted to result in a domination of monoglyceride in the reaction mixture. The curves for percentage of monoglyceride in the glyceride mixture in relation to pH are seen in Fig. 9 and are highly reminiscent of a dissociation curve with a pK of approximately 6.8.

**The Secondary or 2-Ester Bond**

As was mentioned in the Introduction the over-all result of pancreatic lipase action on long-chain triglycerides is complete hydrolysis. This is due to the irreversible splitting of the monoglyceride, the water-soluble glycerol being lost from the site of enzyme action.

The 2-monoglyceride can be expected to be hydrolyzed either directly or after isomerization to the 1-form, which is easily available for the pancreatic lipase (15). At equilibrium the proportion of 1- and 2-monoglyceride is 88 to 12 (21) and the rate of isomerization is pH-dependent, being rapid at alkaline pH (22). Under slightly acid conditions and in the presence of bile salt the rate of isomerization is a first-order reaction (see Fig. 10, replotted from the data of Hofmann (26)). If not enzyme catalyzed—and so far no evidence has been brought forward for the presence of any monoglyceride isomerase in pancreatic juice (17)—the fractional rate of isomerization would be expected to be only time-dependent. In the experiments illustrated in Fig. 11 triolein has been hydrolyzed with pancreatic lipase at three different enzyme levels. At a rather constant level of monoglyceride, the splitting of the monoglyceride is directly proportional to time of incubation (except for the shortest time intervals) and also to the concentration of enzyme. With the highest enzyme concentration used, over 50% of the triglyceride glycerol present from the beginning has been hydrolyzed to glycerol in 160 min. The amount of 2-monoglyceride isomerized during this time to 1-monoglyceride followed by hydrolysis to glycerol could be expected to be less than 10% under these conditions. It would therefore seem that the main fraction of the 2-monoglyceride is directly hydrolyzed by pancreatic lipase during the conditions of the experiments, for if the isomerization of the 2-monoglyceride is not enzyme catalyzed the rate would not be expected to be affected by the removal of 1-monoglyceride formed.

From the initial rates of disappearance of the triglyceride and the appearance of free glycerol at the low enzyme level it can be roughly estimated that the 2-mono-
glyceride is hydrolyzed at a rate that is less than 2% that of the primary ester bonds.

The hydrolysis of the 2-ester bond could a priori also be assumed to take place at the tri- or diglyceride stage. However, the identification of 1,2-diglyceride and 2-monoglyceride as the main glyceride isomers during pancreatic lipolysis (1-3) speaks against such an assumption. It was further shown in the present study that pancreatic lipase could not catalyze the hydrolysis of the 2-ester bond to any significant extent in compounds which had ester bonds in the 1,3-position that were not hydrolyzed by pancreatic lipase (2-oleoyl-oxy-malonic acid-1,3-octadecenyl diester), or which had ether linkages in the 1,3-position (1,3-di-O-dodecyl-2-oleoyl-glycerol). Both these compounds were labeled in the oleoyl moiety; after incubation for 1 hr at pH 6.3 in the presence of 12 μEq of NaTDC per ml and 1.0 ml of lipase solution, <0.4% of the original activity was present in the oleic acid fraction isolated by TLC.

Physiological Implications
Pancreatic lipase exerts its physiological function in the lumen of the proximal small intestine, i.e., duodenum and first part of jejunum. It is clear from the foregoing in vitro studies that several factors operate to determine the course of pancreatic lipolysis in vivo. Of these factors some are known, such as pH, which in duodenum and upper jejunum (the site of fat absorption in the human) is about 6.3 (7), and bile acid concentration, which has been found to be 1.5-10 μEq/l (23). The two other most important factors, which are not well...
known at the present time, are the level of lipase in intestinal content and the time of digestion. These two last parameters should be of importance especially with regard to the extent of total hydrolysis of the triglyceride, i.e. including the hydrolysis of the 2-ester bond. To get information on this point the experiments reported in Table 1 were undertaken.

Normal human beings were intubated with a thin polyvinyl tubing and intestinal content was collected from duodenum or upper jejunum during digestion of a low-fat test meal or a similar test meal containing 55 mg of triglyceride per milliliter (7). Samples obtained after feeding the low-fat test meal were incubated for 1 hr at 37° with 32 pEq of glycerol-labeled triolein, and samples from the fat-rich test meal with trace amounts of labeled triolein (Table 1). These results show that intestinal content under these conditions effects the total hydrolysis of triglyceride to an extent of 12-27% and also seen that the extent of hydrolysis to free glycerol is almost linear with time (Table 2).

No exact figure for the time factor in normal digestion is available at the present time. The 1 hr period of digestion used here in vitro probably is well above the maximal digestion in vivo, and therefore the extent of hydrolysis to glycerol under normal conditions is proportionally less than the above figures.

These results are in agreement with those showing that 80-90% of dietary glycerides retain their fatty acid in the 2-position during the entire digestion and absorption process [also including any intracellular hydrolysis (24, 25)].

It is, however, also clear that the lipase level in human intestinal content is such that in cases where absorption is for some reason retarded, a considerable hydrolysis of the monoglyceride could occur. This is important, as it has been shown that the monoglycerides are necessary for the prevention of calcium soap formation (26).

From the amounts of glycerol produced from triolein during in vitro incubation with intestinal content (compared to pancreatic lipase) it seems fair to conclude that the lipase content of intestinal juice is high enough to catalyze in a relatively short time a steady state as regards synthesis and hydrolysis of the primary ester bonds. This is in agreement with the results of earlier studies of the exchange of free fatty acids and glyceride fatty acids during digestion in the human (27). Such a steady state system of glycerides and fatty acids catalyzed by pancreatic lipase has interesting physiological properties.

In a closed system, i.e. in vitro, there will be no net change in the steady state except that caused by a slow hydrolysis of the 2-monoglyceride. The system is, however, capable of generating any of its products that are removed from the system. Other work has shown that intestinal content during digestion is a detergent solution which can dissolve monoglycerides and fatty acids, but only to a very limited extent di- and triglycerides, in micellar solution (18, 26). It has also been demonstrated in vitro that rings of rat small intestine accept monoglycerides and fatty acids from micellar bile salt solutions, but take up emulsified triglycerides only to a limited extent (28, 29). The physiological function of the lipase catalyzed equilibrium state in intestinal content would therefore be the continuous generation of monoglycerides and fatty acids to the brush-border of the mucosal cells.

The occurrence of such an equilibrium system of glycerides and fatty acids will also explain the rather constant composition of the glyceride mixture found in intestinal content collected from the site of absorption of fat in the human (30).

This work was supported in part by P.H.S. Research Grant HE-05302, The National Institutes of Health, U.S. Public

### Table 1: Per Cent of Triglyceride Glycerol Hydrolyzed to Free Glycerol from Glycerol-14C Trioleate during Incubation with Human Intestinal Content

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Free Glycerol</th>
<th>Total Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>mg/ml of intestinal content</td>
</tr>
<tr>
<td>1</td>
<td>25.5</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>20.8</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>27.2</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>17.1</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>19.4</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>19.8</td>
<td>25.0</td>
</tr>
<tr>
<td>7</td>
<td>16.1</td>
<td>24.4</td>
</tr>
<tr>
<td>8</td>
<td>25.2</td>
<td>4.0</td>
</tr>
<tr>
<td>9</td>
<td>20.2</td>
<td>9.0</td>
</tr>
<tr>
<td>10</td>
<td>10.8</td>
<td>29.1</td>
</tr>
<tr>
<td>11</td>
<td>14.0</td>
<td>60.7</td>
</tr>
</tbody>
</table>

Glyceryl trioleate (32 μEq) was incubated in vitro for 1 hr at 37° with the intestinal content, which was collected from distal duodenum-proximal jejunum of two normal human beings intubated with a polyvinyl tubing. They were fed either a fat-free (1-5) or a fat-rich (55 mg/ml) test meal (6-11) and fractions were collected for 30-min periods.

### Table 2: Time Course of Free Glycerol Formation from Glycerol-Labeled Triolein Incubated with Human Intestinal Content

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>Free Glycerol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>4.7</td>
</tr>
<tr>
<td>30</td>
<td>9.8</td>
</tr>
<tr>
<td>45</td>
<td>14.1</td>
</tr>
<tr>
<td>60</td>
<td>16.7</td>
</tr>
<tr>
<td>120</td>
<td>31.7</td>
</tr>
</tbody>
</table>

Glyceryl-C14 trioleate (32 μEq) incubated at 37° with 4 ml intestinal content collected during digestion of a fat-free test meal.
Health Service; The Swedish Medical Research Council; and the Association of the Swedish Margarine Industry.

Manuscript received December 13, 1963; accepted April 16, 1964.

REFERENCES