Enzymatic Hydrolysis at an Oil/Water Interface

F. H. MATTSON and R. A. VOLPENHEIN,
The Procter & Gamble Company, Miami Valley Laboratories, Cincinnati, Ohio

Abstract

Triglycerides are metabolized by most of the organs and tissues of the body. However, the conditions in the lumen of the intestinal tract are unique, for it is only here that triglycerides are metabolized in the free state. Elsewhere these lipids are associated with water-soluble materials. Since the substrate is water-insoluble and the enzyme is water-soluble, lipase, which brings about the hydrolysis of triglycerides in the intestinal tract, has the special property of being capable of functioning efficiently at an oil/water interface. Any material that can alter the nature of this oil/water interface can markedly influence the digestion of triglycerides.

Because of the unusual conditions under which the hydrolysis of triglycerides occurs, the usual methods of studying enzyme kinetics are not applicable. Besides the enzyme-substrate reaction itself, one must consider also such matters as diffusion of the substrate to the oil/water interface, removal of the products of hydrolysis from the oily/water interface, and the subsequent diffusion of these into the bulk phase. All of these steps can be influenced by such variables as efficiency of agitation, electrolyte concentration, and the presence of surface-active agents, such as monoglycerides, soaps, and the bile salts.

Introduction

THE ELUCIDATION of the mechanisms by which triglycerides are hydrolyzed in the intestinal tract poses a particular challenge. Not only is it necessary to make the usual characterizations of an enzymatic reaction but also to resolve the added and sometimes separate problems that exist because these reactions take place at an oil/water interface. This discussion is primarily concerned with the physical properties that can be important in this hydrolysis because of its occurrence at an interface, and not with the enzymatic reaction itself. For this purpose, we will not present a review of the literature nor an extensive amount of new experimental evidence. Rather we have selected from these sources examples to illustrate the nature of the reactions at an oil/water interface by which glycerides are hydrolyzed.

The route of hydrolysis of triglycerides is now adequately established as being a series of directed step-wise reactions from triglyceride to 1,2-diglyceride to 2-monoglyceride (1). The further hydrolysis of 2-monoglyceride likely occurs after isomerization to 1-monoglyceride. These reactions are brought about primarily, if not entirely, by the enzyme lipase in pancreatic juice.

Animals have a great capacity for hydrolyzing triglycerides. For example, an adult rat produces about 25 ml of pancreatic juice per day. We have assayed this for enzymatic activity, and found that 1 ml is capable of hydrolyzing 120 mg of oleate ester per minute. Thus, the rat has the potential of hydrolyzing about 75 g of fat per day.

Enzyme-Glyceride Complex

This ability to hydrolyze triglyceride is impressive, but it is even more remarkable that this enzyme cannot hydrolyze a water-soluble substrate. This is well illustrated by the experiments of Sarda and Desnuelle (2). Using methyl butyrate as a substrate, they demonstrated that as long as its solubility in water was not exceeded, it was hydrolyzed little, if at all, by pancreatic lipase. However, when the solubility limit was exceeded, the substrate was hydrolyzed rapidly. Thus, pancreatic lipase has the unusual property of being functional only at an oil/water interface.

It is because the reaction takes place at an oil/water interface that a number of factors, not ordinarily considered in enzyme reactions, become important. Simulation in vitro of digestion poses special problems. Even some of the simplest changes in the environmental conditions can markedly influence the reaction. Since this is a reaction in which the enzyme is soluble in water, whereas the substrate is insoluble in this medium, one important new factor is the rate at which the reactants diffuse to the interface and the rate at which the products diffuse away from the interface.

The importance of diffusion can be illustrated by a simple experiment. We carry out our digests on a wrist-action shaker that has an amplitude of 25°. A rate of shaking of 150 cycles/min imparts considerable agitation to the system, yet the rate of hydrolysis is only half that realized when shaking is at a rate of 330 cycles/min. The agitation condition we have selected for assay is that which gives a rate of hydrolysis that is not increased by an increase in the rate of shaking. Most stirring devices do not give sufficient agitation to permit the optimum rate of hydrolysis.

In the hydrolysis of triglycerides the first of the diffusion steps is that which brings the enzyme and substrate together. This is a diffusion of the enzyme to the substrate. Benzonana and Desnuelle (3) have found that there is an actual adsorption of the enzyme at the oil/water interface, and a depletion of the enzyme from the bulk aqueous phase. This affinity of the enzyme is apparently for an interface rather than specifically for triglycerides because Baskys, Klein and Lever (4) used adsorption of the enzyme at an ether/water interface as the initial step in purifying lipase.

For the enzyme to act on the triglyceride, the ester linkage must be so located at the oil/water interface that it is available to the enzyme. The exact orientation of a triglyceride at an oil/water interface is not known. However, it is reasonable to assume that the more hydrophillic nature of the ester linkages and the interactions among the hydrocarbon chains by van der Waal forces would result in the glycerol of the triglyceride molecule lying on the surface of the fat droplet with the hydrocarbon chains extended toward the center of the droplet.

Fatty Acids in Water

Diffusion once again becomes important in the removal of the free fatty acid that results from the hydrolysis of triglyceride to diglyceride. It has long been recognized that free fatty acids are inhibitors of the hydrolysis. It is reasonable that this action is

due to their accumulation at the interface, thus blocking the formation of the enzyme-substrate complex and consequently slowing the reaction.

Determining the mechanism of free fatty acid removal is complicated by the unusual properties of water-insoluble fatty acids in aqueous systems. The titration curve for acetate, a water-soluble acid, in Figure 1 shows this compound to have a pKa of less than 5. It has been shown that longer chain fatty acids, if they are water-soluble, have about this same pKa. The addition of sodium chloride to a solution of water-soluble fatty acids lowers the activity coefficients of the ions and thus decreases the pKa about 0.1 unit. In Figure 1 an entirely different situation is found in the case of the water-insoluble acid, oleate. Although the true pKa of a fatty acid is about 5, this acid has an apparent pKa of about 9. The addition of NaCl causes a marked shift of the apparent pKa to lower values.

This apparent pKa of about 9 for water-insoluble fatty acids was reported in the biochemical literature a number of years ago by Schmidt-Nielsen (5). Since then, with one or two exceptions, this phenomenon has been ignored. One explanation of these titration curves that he advanced involved the formation of acid-soaps. The simpler explanation, which satisfactorily explains the observations, is that the phenomenon is due to hydrolysis of soaps in which

$$\text{Na oleate} + \text{HOH} \rightleftharpoons \text{Oleic acid} + \text{NaOH}$$

However, one of the products, oleic acid, is insoluble in water and as a consequence the reaction is driven in the direction of hydrolysis. The presence of salt shifts the steady state that is attained to the left.

The fatty acids released from triglycerides during hydrolysis in vitro similarly show a pattern of soap to nonesterified fatty acid ratio as a function of pH. This can be demonstrated by carrying out the digests at various pH values with the selected pH maintained by the addition on demand of standardized KOH. After 10 min, the volume of KOH added in this aqueous titration is recorded. The digest is acidified and the lipids are extracted. The total free fatty acids in this lipid extract are determined by titration in alcoholic solution. The ratios of KOH added during the aqueous titration (soap) and that used in the titration in alcohol (total nonesterified fatty acid) when the digest was carried out at various pH values are given in Figure 2. The curve is similar to that for oleate in Figure 1. The half-equivalence value yields an apparent pKa of 7.8. Note that of the nonesterified fatty acids formed during digestion, 90% of these is soap at pH 9, while at pH 7 only

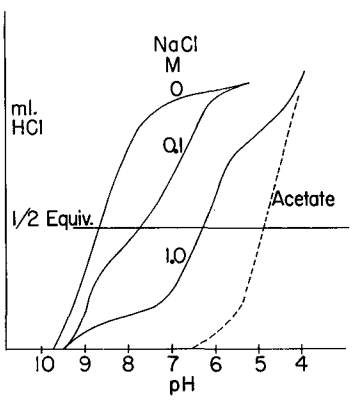

FIG. 1. Titration curves of sodium oleate dissolved in 0, 0.1, and 1.0 M aqueous NaCl and of sodium acetate in water.

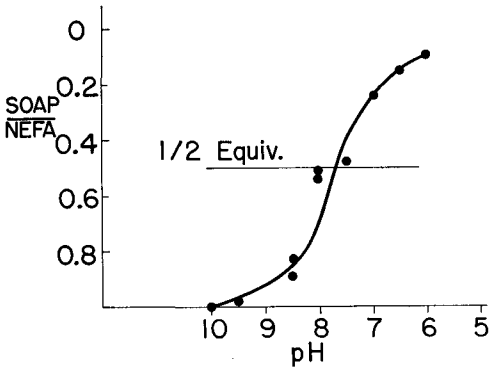

FIG. 2. Ratio of soap to total nonesterified fatty acid (NEFA) found in enzymatic digests of triglycerides carried out at various pH values. Digestion conditions: 200 mg triolein; 1 M NaCl; 0.3 mg lyophilized rat pancreatic juice; total volume, 55 ml; pH maintained with pH stat; digested for 10 min at 25C.

20% is present as soap. Since soap and free fatty acid do not have the same surface active properties, it is apparent that the hydrolysis of glycerides, which occurs at an oil/water interface, will be influenced by a change in the pH at which the digestion is carried out simply because of the change in the ratio of soap to nonesterified fatty acid.

In connection with the property of fatty acids in aqueous systems, the correlation of the conversion of triglycerides to monoglycerides with an acid having a pKa of 6.8 is interesting (6). This could well be a reflection of the phenomenon described above. An incidental question that deserves raising is the form in which nonesterified, water-insoluble fatty acid occurs in blood. Although most of it is bound to albumin (7), the work of Spector and Steinberg (8) suggests that it is the unbound form that is taken up by the cell. If the titration curves in Figure 1 are applicable then, in spite of the slightly alkaline pH of blood and a true pKa of about 5, such unbound fatty acid will not be present as soap but primarily as unionized fatty acid.

$$\text{Diglyceride} \longrightarrow \text{Monoglyceride}$$

When triglycerides are hydrolyzed by lipase, there is an initial accumulation of diglycerides; subsequently monoglycerides are formed (9,10). Borgström (11) has shown that both the triglyceride to diglyceride and diglyceride to monoglyceride steps are reversible. These observations must mean that the enzyme-partial glyceride complex is broken after each hydrolytic step and a new association of that molecule of enzyme with a triglyceride or a diglyceride molecule is formed. Thus, each hydrolytic step involves not only a diffusion of the resulting free fatty acid but also a diffusion of the enzyme to a new site at the oil/water interface.

As in the case of triglycerides, the orientation of diglycerides at an oil/water interface is not known with certainty. The results in Table I, taken from a paper by Hartman (12), show that, in spite of the presence of a free hydroxyl group, diglycerides have no stronger tendency to accumulate at an oil/water

TABLE I

Interfacial Tension in Dynes/cm at 50C of Oil-Water Systems with Added Glycerides (0.5% Partial Glyceride in Soybean Oil) (12)

Soybean oil control	29.6
+ 0.5%	
1-Monostearin	22.4
2-Monostearin	21.4
1,3-Distearin	29.0
1,2-Distearin	28.6

interface than do triglycerides. On the basis of structure, one would expect diglycerides to be more surface active than triglycerides. Possibly the experimental method is not sensitive enough to detect this. It is likely that the orientation of diglycerides at the oil/water interface is similar enough to that of triglycerides that the same physical factors that influence the hydrolysis of triglycerides to diglycerides are equally operative in the conversion of diglycerides to monoglycerides.

Role of Bile

One of the important problems that remains only partly solved is the role of bile in the digestion of fat. The marked surface-active properties of bile, early led to the obvious conclusion that the function of bile was to emulsify the water-insoluble substrate and thereby make it available to the water-soluble enzyme. In vivo, triglycerides can be hydrolyzed in the absence of bile. The possibility remains that bile speeds the rate of the reaction. Our experience has been that bile is an accelerator only under certain very limited conditions. Under all other conditions, it has either no effect or, more often, it is inhibitory.

Several years ago Borgström (13) suggested that one of the functions of bile is to lower the pH optimum of lipase so that it corresponds to that found in the intestinal tract. In Figure 3, two of his curves have been redrawn. First, it should be noted that when the digestion is carried out at pH 8, bile salts have only an inhibitory action. At pH 6, the addition of bile salts up to a level of 0.2% causes an acceleration in the rate of hydrolysis. However, higher concentrations do not show this accelerating action. The concentration of bile salts in the intestinal tract is between 0.1 and 1.0% (14). Thus, only at the lowest level would bile salts have any marked accelerating effect.

There are numerous papers in the literature, including one of our own (10), reporting that bile accelerates the hydrolysis of triglycerides. However, these experiments are not necessarily conclusive. For example, there is the relationship between agitation and the role of bile salts which has been pointed out by Frazer (15). Moreover, most experimenters have used either a commercial bile preparation or one of the individual bile salts. Figure 4 presents results obtained in our laboratories for the hydrolysis in vitro of triglycerides by lyophilized rat pancreatic juice in the presence of various amounts of lyophilized bile juice from a rat. At all levels, the bile caused only inhibition. For the present, our opinion is that the

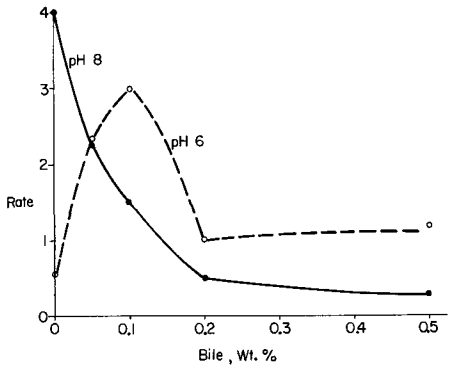

FIG. 3. The rate of hydrolysis of triglycerides at pH 6 and 8 in the presence of various amounts of bile. Redrawn from Borgström (13).

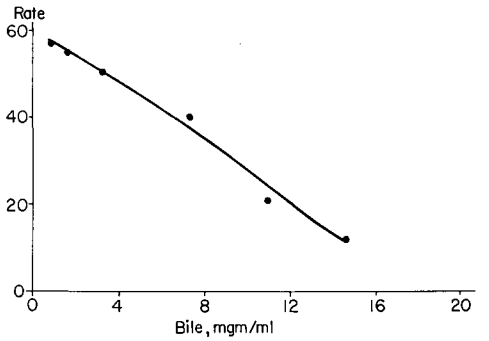

FIG. 4. The rate of hydrolysis of triglycerides at pH 6.5 by rat pancreatic juice in the presence of various amounts of rat bile. Digestion conditions: 200 mg triolein; 1 M NaCl; 0.1 M histidine; pH 6.5; 0.3 mg lyophilized rat pancreatic juice; lyophilized rat bile as indicated; total volume 55 ml; digested for 10 min at 25C. Free fatty acids determined by acidification, extraction, and titration in alcohol.

role of bile in the digestion of triglycerides has not been established with certainty. It is striking that under many conditions bile inhibits the reaction.

$$\text{Monoglyceride} \longrightarrow \text{Glycerol}$$

The experiments of Hofmann and Borgström (16, 17) led them to assign two other roles to bile. The first of these is the formation of mixed micelles containing free fatty acid, monoglyceride, and bile salt. Although this phenomenon has been emphasized for its role in absorption, it may be that an important accompanying effect is the removal of monoglyceride from the oil/water interface, another step where diffusion is important. Such an alteration in the diffusion of monoglyceride would offer an alternative explanation for the equilibrium reported by Borgström (6) between the hydrolysis of triglyceride to monoglyceride and the synthesis of triglyceride from monoglyceride. The function of the surface active bile salts then would not be to disperse the insoluble triglycerides but rather to remove the surface active monoglycerides, which orient themselves at the oil/water interface, and if allowed to accumulate would prevent the hydrolysis of other triglycerides and diglycerides.

The hydrolysis in vitro of monoglycerides takes place at a much slower rate than does the hydrolysis of triglycerides and of diglycerides. However, an appreciable amount of free glycerol is one of the products formed in the living animal (18). The specificity of lipase for esters of primary alcohols, mitigates against its directly splitting 2-monoglycerides. However, 2-monoglycerides isomerize to 1-monoglycerides in aqueous systems (19), although at a slower rate in the presence of bile (20). Thus, a pathway for the formation of free glycerol is by the hydrolysis of 1-monoglyceride that has been formed by the isomerization of 2-monoglyceride.

In vitro, 1-monoglycerides are hydrolyzed very slowly, if bile is not present. This lessened rate is probably attributable to the marked orientation of monoglycerides at the oil/water interface. The extension of the two free hydroxyl groups into the aqueous phase conceivably blocks the enzyme from the ester linkage. However, as Hofmann and Borgström (16) have shown, the addition of bile salts permits the hydrolysis of monoglycerides by pancreatic lipase. Their observation that hydrolysis is initiated below the critical micelle concentration of the system eliminates the possibility that hydrolysis occurs when the substrate is in form of a micelle.

These results point to at least two possible functions of bile salts. One, at and above the critical micelle concentration, involves the removal of monoglycerides from the oil/water interface. The other, at concentrations below the critical micelle concentration, permits the hydrolysis of 1-monoglycerides, either by direct effect on the enzyme or by altering the oil/water interface.

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