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Review

Specificities of lipases

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I. INTRODUCTION

Until an enzyme has been obtained in a pure state, it cannot be directly characterized. This is obvious from a quantitative point of view. We cannot weigh an impure enzyme, and we are obliged, therefore, to take the magnitude of its catalytic activity as a measure of quantity. In other words, we determine enzymatic units, which, under appropriate conditions, are proportional to the amount of enzyme.

The same idea should also be carefully considered from the qualitative point of view. It is difficult to identify an impure enzyme by any technique of organic chemistry. We discover its presence in a given preparation because this preparation catalyzes a specific reaction. Thus, many enzymes must be presently defined by the reactions they catalyze. Clearly, this kind of definition would be valueless if one enzyme catalyzed several reactions. That is one reason why the specificity concept is so important in enzymology. Another reason is that full characterization of an enzyme in vitro enables better understanding of its function in vivo.

Esterases comprise a broad and rather ill-defined family of enzymes hydrolyzing ester bonds in various substrates. Within this family, it is customary to distinguish lipases, but the borderline between esterases proper and lipases has never been drawn with absolute certainty (1–3). The differentiation is sometimes founded upon the chemical nature of the substrate, lipases being more or less associated with the degradation of typical triglycerides. In other instances, it is founded upon the length of the acyl chain of the ester substrate, lipases splitting preferentially esters of longer-chain fatty acids, and esterases splitting preferentially esters of shorter-chain fatty acids. The physical state of the substrate and the nature of the inhibitors have also been suggested as differentiating features.

This review is devoted to the specificities displayed by the best characterized lipases, mainly pancreatic lipase.

II. SUBSTRATE SPECIFICITY

By substrate specificity is understood the influence exerted on lipase activity by the physical state and chemical nature of the substrate.

PANCREATIC LIPASE

1. *General Considerations.* Pancreatic acinar cells synthesize an enzyme called pancreatic lipase, which flows with pancreatic juice into the duodenum where it promotes the hydrolysis of dietary triglycerides. Therefore, the safest way to investigate this enzyme in vitro is to use as substrate long-chain, water-insoluble triglycerides in an emulsified state (4, 5).

The use of emulsions containing multimolecular substrate globules is the source of many difficulties in enzymatic tests. The mechanism by which water-soluble lipase associates with the emulsified globules and the role played in this association by the emulsifier itself and the "activators" are still poorly understood. Nevertheless, as shown later in more detail, it now seems fairly well established that pancreatic lipase should be tested on emulsified esters (4–6). When the ester substrate is soluble in water, it is not attacked by lipase, but, rather, by another enzyme, or enzymes, present in the pancreas.

Several techniques have been recently proposed for determining lipase activity with triglyceride emulsions. The hydrolysis of the ester bonds may be followed by titrimetry (7, 8), manometry (9), or, in a more indirect way, nephelometry (10, 11). The emulsifier may be gum arabic (7), gum acacia (4), polyvinyl alcohol (8), or monoglycerides (11). No emulsifier is added in the manometric technique using tributyrin as

substrate (9). Figure 1 gives results obtained by a potentiometric technique (7) in which the liberated fatty acids are titrated at a constant pH. The technique may be made fully automatic by the use of a recording pH-stat (12, 13). It is seen that, despite the heterogeneous nature of the emulsion, linear kinetics are obtained from which initial rates can be easily calculated. The slopes of the straight lines are proportional to the enzyme amounts within large limits.

The best known of all pancreatic lipases is porcine lipase. After many difficulties caused by using substrates dissolved in water, this enzyme was recently purified by a combination of fractional precipitations and preparative zone electrophoresis (7, 14–17). It can also be partially purified by chromatography on diethylaminoethyl-cellulose columns with a concentration gradient of phosphate buffer at pH 8.0 (18) or by stepwise elution at the same pH (19). Other lipases have been characterized by enzymatic assays or by chromatographic fractionation of pancreatic tissue extracts and pancreatic juices of various species. Cattle (20, 21), dog (18, 21), rat (11, 19, 21), and human

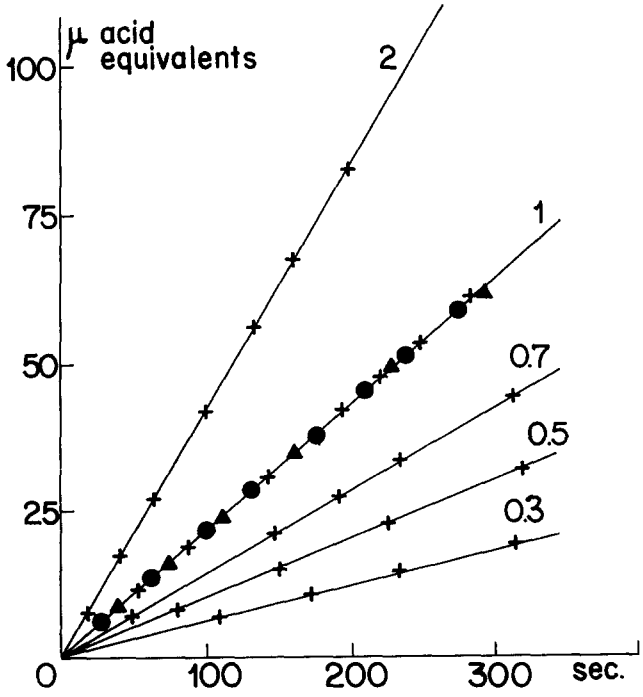

FIG. 1. Determination of lipase activity by the potentiometric method at constant pH (7). The good fit of experimental points (crosses, black circles, and triangles along curve 1) demonstrates the reproducibility of the test. The slopes of the curves are proportional to the relative amounts of lipase used in each experiment (0.3, 0.5, 0.7, 1, and 2). The substrate is a specially prepared emulsion of long-chain triglycerides in 10% gum arabic. The amount of emulsified phase is very large in order to obtain maximal rates. The pH is maintained at 9.0 by addition of sodium hydroxide. The temperature is 37°.

(21) pancreas contain relatively large amounts of lipases still awaiting purification.

2. *Physical State of the Substrate.* As already stated, the biological role of pancreatic lipase is to promote intraluminar hydrolysis of long-chain triglycerides. Thus, lipase should have the rather unusual property of being able to act at a very high rate on substances insoluble in water. A widespread idea is that some of the activators commonly used during lipolysis, such as bile salts, in some way "solubilize" the natural substrates of lipase. However, the following experiments demonstrate that lipase does not normally attack esters in aqueous solution (6).

Let us first consider the experiments described in Fig. 2. Both diagrams of the figure indicate the activity of a given amount of lipase as a function of the amount of substrate. In this respect, they may be considered as Michaelis curves. The substrates (triacetin on the left, methyl butyrate on the right) have been selected for their limited solubility in water. At low concentrations, they give true solutions. When more substrate is added, the solutions become more concentrated and, finally, saturation is reached. This point of saturation is indicated in the figure by a vertical dotted line. On the right of this line, additional ester molecules no longer dissolve. Rather, they appear in a new physical state that may be called, for the time being, an emulsion. When still more substrate is added, the amount of this new form increases.

The curves of Fig. 2 show that impure lipase displays a weak activity against triacetin and methyl butyrate solutions. However, this kind of activity disappears completely (methyl butyrate) or almost completely (triacetin) when more purified preparations of lipase are used. In contrast, as soon as the substrate solutions are oversaturated, a very large lipolytic activity appears. This second kind of activity is not materially lowered by the purification procedure.

Since monoalcoholic esters, such as methyl butyrate, are rather poorly split by pancreatic lipase (see later), the negative results obtained with this type of compound do not prove that the enzyme is completely unable to attack the dissolved substrate. Indeed, experiments performed with triacetin and other, still unpublished, experiments suggest that even very pure lipase exhibits a weak activity against substrates in solutions. But the important point is the large increase in activity that occurs when the substrate solution becomes oversaturated.

This highly interesting property is not shown by typical esterases. When purified liver esterase, for example, is assayed against increasing concentrations of methyl butyrate, enzymatic activity begins at low

concentrations and does not increase disproportionately when the substrate solution becomes oversaturated (6). Thus, pancreatic lipase and liver esterase have entirely different specificities with regard to the physical state of the substrate. Liver esterase acts, as most enzymes do, on aqueous solutions of their substrates. Pancreatic lipase, on the other hand, is an unusual enzyme that does not act, or acts very weakly, on dissolved ester molecules, but that becomes able to act on the same molecules when oversaturation induces some kind of molecular aggregation. The right state for lipase action apparently exists, not only in supersaturated solutions of soluble esters, but also in emulsions directly prepared with insoluble esters, such as long-chain triglycerides.

Another interesting point is that, for a fixed amount of enzyme, lipase activity increases with the amount of emulsified phase. This increase is shown in Fig. 2 by the curves on the right of the dotted lines. Similar curves, starting from the origin, are obtained with more or less "concentrated" emulsions of insoluble triglycerides (6). These curves look like typical Michaelis curves. After an initial rise, lipase activity reaches an upper limit proportional to the amount of enzyme used.

The nature of the physical state inducing lipase activity is still unknown. However, a rather simple assumption is to consider that emulsions contain globules or multimolecular aggregates separated from water by an interface, and that the interface determines lipase action by adsorbing the enzyme. When no interface exists (e.g., in true solutions), lipase would be dissolved in water and inactive. In the presence of a very large interface, all the available lipase molecules would be adsorbed and active. In all other cases, the enzyme would be partitioned between water and the interface, and, therefore, partially active.

The proportions of adsorbed lipase can be experimentally determined by centrifuging the emulsions and measuring the lipolytic activity of the clear aqueous phase lying under the cream. In this way, it may be verified that the adsorption isotherm at pH 9.0 of lipase as a function of increasing amounts of emulsified phase is almost identical with the activity curve at the same pH (22). Thus, a good correlation appears to exist between lipase adsorption and lipase activity. The activity curves on the right of the diagrams of Fig. 2 describe, as in the usual case of soluble substrates, the progressive formation of the enzyme-substrate complex. But, in the specific case of lipase, this formation is due to the adsorption of the enzyme on the insoluble substrate and not to the adsorption of the substrate on the enzyme.

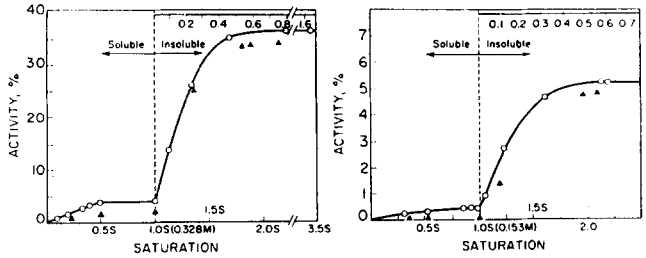

FIG. 2. Activity of porcine lipase as a function of the amount of substrate. Case of an ester soluble in water (6). Ordinates, activity of a given amount of lipase at pH 9.0 and 37°, in percentage of maximal activity on triolein. Abscissae, amount of substrate (triacetin on the left, methyl butyrate on the right) expressed in fractions of the quantity that makes a saturated solution at the temperature of the experiment. On the right of the vertical dotted line, the solutions become oversaturated and the values become higher than unity. Open circles are experimental points obtained with an impure preparation containing "esterase" as well as lipase activity. Black triangles are points obtained with a purified preparation, from which the "esterase" activity has been eliminated by zone electrophoresis in starch (see Fig. 3).

What happens after the adsorption of lipase is another problem. Do the enzyme molecules remain at the interface of the emulsified globules where they find, at the same time, the substrate on one side and water on the other? If this is correct, lipase action would be a typically heterogeneous process. Do lipase molecules penetrate into the globules and then act in a homogeneous phase containing the substrate and dissolved water molecules? An experimental approach to this problem would be to determine whether lipase activity and adsorption are governed by the area of the interface or by the weight of the emulsified phase (22). It would also be interesting to see whether electrostatic factors play a role in the formation of the lipase-substrate complex, as they do, for instance, in the case of phospholipases. Owing to technical difficulties, none of these questions has yet been satisfactorily answered.

The very weak activity displayed by lipase against isotropic micellar dispersions of 1-monoglycerides and bile salts (23) may perhaps be related to the low activity against true solutions of triacetin.

The preferential action of pancreatic lipase on emulsified esters is again stressed by Fig. 3, which gives an electrophoretic diagram (24) of an impure preparation of lipase. The activities of the fractions eluted from the column after electrophoresis have been tested on both emulsified *and* dissolved esters. It is seen that the lipase peak, under which all activities against emulsified esters are found, is clearly separated from the peak containing the activities against dissolved esters. This diagram shows that lipase activity should be measured by using an ester *emulsion*, and

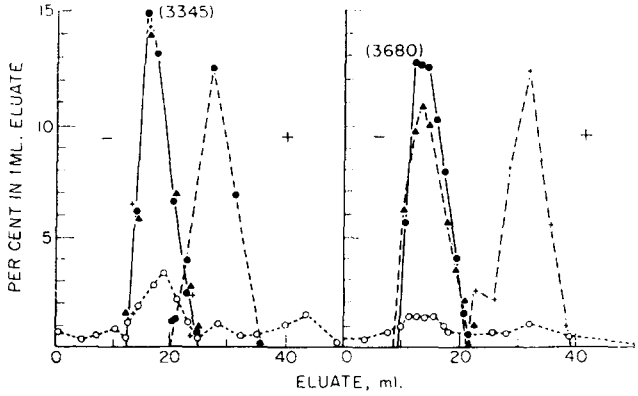

FIG. 3. Electrophoretic separation of pancreatic lipase and "esterase" (24). A partially purified preparation from porcine pancreas is subjected to zone electrophoresis of long duration in a starch column equilibrated with 0.025 M acetate buffer at pH 5.25. The fractions eluted from the column at the end of the process are tested on (1) *emulsions* of triolein (black circles and solid line, both diagrams), tributyrin (crosses, left diagram), methyl oleate and laurate (black circles, left diagram), and *p*-nitrophenyl laurate (black triangles, right diagram); (2) *solutions* of methyl butyrate (dark circles and dotted line, left diagram), and *p*-nitrophenyl acetate (crosses and dotted line, right diagram). All the activities against emulsions are found under the same peak, nearly isoelectric at pH 5.25. The activities against solutions are under another peak migrating toward the anode.

not an ester *solution*, which can be hydrolyzed by ill-defined "esterases."

It is perhaps useful to recall here that the problem of pancreatic "esterase" or "esterases" urgently needs clarification. On one hand, the carboxylic or phenolic esters (methyl butyrate, valeryl salicylate, *p*-nitrophenyl acetate, etc.) employed for characterizing this kind of activity are not normally present in the intestine. Most of them can be hydrolyzed by proteolytic enzymes such as trypsin, chymotrypsin, and an enzyme originating from procarboxypeptidase-A. Moreover, the esterase recently crystallized from porcine pancreas (25, 26) exists, like typical proteolytic enzymes, in the form of an inactive precursor named proesterase, and it exhibits proteolytic as well as esterolytic activity. Thus, the possibility must be considered that at least some of the so-called pancreatic "esterases" merely correspond to side-activities of well known proteolytic enzymes. In any case, these activities should not be confused with lipase activity.

A final observation concerning the physical state of lipase substrates is that these should be liquid at the temperature of the experiment. Long, saturated fatty chains, such as lauryl, palmityl, and stearyl chains, are very slowly hydrolyzed at 37° when they occur in high melting trisaturated glycerides. In contrast, the same chains are easily split in mixed-acid glycerides of lower melting point. It is also known that lipase is much better adsorbed by an emulsion of liquid triolein than by a crystalline suspension of tristearin.

3. *Chemical Nature of the Substrate.* The above experiments suggest that pancreatic lipase may attack any ester, provided that it is in the proper physical state. But the chemical nature of the substrate is also a factor that cannot be neglected. The influence on lipase action of the two moieties (the fatty acyl moiety and the alcohol moiety) linked by the ester bond is discussed in the following sections.

(a) *The Acyl Moiety.* The influence of the chemical nature of the chain may be investigated by several techniques. The first is to use a series of triglycerides containing three identical chains (triacetin, tripropionin, tributyrin, etc.) or a series of mono esters (methyl acetate, methyl propionate, methyl butyrate, etc.), and to determine in each case the parameters K_m and V_{max} of their hydrolysis by a fixed amount of lipase under a given set of conditions. The synthesis of these compounds is easy. But the difficulties arise from the fact that a number of different rates of reaction taking place in the poorly defined emulsions have to be compared. Also, the general characteristics of the emulsions may then have a much stronger influence on the rate than the chemical nature of the substrate. A striking illustration of this situation is given in the case of tristearin. Tristearin is a poor substrate for lipase, not because of the chemical nature of stearyl chains, but because of its high melting point. Moreover, the emulsifier used for stabilizing the emulsions, and various inhibitors or activators, may play an important role. For example, the optimal concentration of bile salts, calcium ions, and hydrogen ions varies a great deal with the length of the acyl chain (27, 28).

No really precise information is available about the K_m of reactions catalyzed by lipase. But the relative rates at which the enzyme splits off different acyl chains have been evaluated several times. When lipase activity is measured manometrically in the absence of an emulsifier (16), the following rate values are obtained: triacetin (C_2), 24; tripropionin (C_3), 100; tributyrin (C_4), 77; tricaproin (C_6), 15; tricaprylin (C_8), 27; trilaurin (C_{12}), 2; tripalmitin (C_{16}); and tristearin (C_{18}), very low.

In another series of experiments (28), pre-formed triglyceride emulsions in gum arabic were used in the presence of optimal concentrations of bile salts. In each case, the amount of the insoluble substrate was such as to give maximal rates (V_m). The variations of this rate with the length of the chain are given in Fig. 4. Here, V_m increases strongly from C_2 (triacetin) to C_4 (tributyrin) and then decreases until C_{10} (tricaprin). Triglycerides containing longer chains

have not been investigated in this way because their melting points are too high. From C_{10} upwards, experimental points obtained by another technique are merely connected by a dotted line in order to give a general picture of the phenomenon. The main conclusion to be drawn is that preferential hydrolysis of shorter chains by lipase seems obvious. The highest rate is found here for tributyrin rather than tripropionin. But this small discrepancy may be easily explained by different experimental conditions. When tributyrin is purified by silicic acid chromatography, the initial rate of its hydrolysis under optimal conditions is found to be about three times as high as for triolein.

A second experimental approach is to compare the rate of hydrolysis of two different fatty acid chains belonging to the same triglyceride molecule. Many difficulties are avoided in this way, but the effects of the positional specificity of lipase (see later) must be eliminated, either by distributing the chains at random on all possible positions, or by synthetizing glycerides in which different chains occupy equivalent positions.

Random distribution is taken to mean that any acyl chain will occur with equal frequency in each of the three positions of the glycerol backbone. It is obtained by interesterification of a mixture of glycerides in the presence of suitable catalysts or by direct esterification of glycerol by a mixture of fatty acids. Once obtained, the interesterified or esterified glycerides are treated by lipase until about 10–20% of the chains are liberated. The free fatty acid fraction is separated and analyzed. Its composition indicates the rate at which various chains have been split.

Two interesting facts have been found in this way. All the so-called long chain fatty acids, namely the saturated chains from C_{12} to C_{18} (lauryl, myristyl, palmityl, stearyl) and the most frequent C_{18} unsaturated chains (oleyl and linoleyl) are removed at about the same rate by lipase (29, 30). The value 100 is attributed to this common rate in Fig. 4. On the other hand, it can be confirmed that shorter chains are liberated more rapidly than longer ones (28). For instance, a mixture containing nearly equal proportions of randomly distributed butyryl and oleyl chains was subjected to a 11% lipolysis at pH 9.0 by purified lipase. The molar composition of the free fatty acid fraction was 76% butyric acid and only 24% oleic acid.

Finally, it will be shown later that lipase does not hydrolyze long fatty acid chains bound to the inner carbon (carbon 2) of the glycerol backbone. But the two outer positions (positions 1 and 3) seem to be equivalent. When pure 1-butyryl 3-palmityl glycerol is hydrolyzed to an extent of 10% by purified lipase, the free fatty acid fraction contains 72 moles of the shorter chain for 28 moles of the longer one (28). In contrast, when the two outer chains of a triglyceride are of similar length, as in 1-oleyl dipalmitin, for instance, these chains are removed at the same rate (31).

Thus, it is very likely that, under a given set of conditions, pancreatic lipase splits esters of shorter-chain fatty acids at a particularly high rate. It is not yet known whether this kind of specificity arises from a better orientation of the shorter chains when the complex lipase-substrate is formed, a quicker transfer of these chains from the complex to the enzyme, or a higher instability at a given pH of the resulting acyl lipase. But its biological significance is clear. It has been repeatedly pointed out that shorter chain fatty acids are carried away from the intestinal region as free fatty acids by portal blood rather than in an esterified form by lymph (32). This special behavior seems to be due to the combined effect of two specificities, the specificity of pancreatic lipase, which quickly

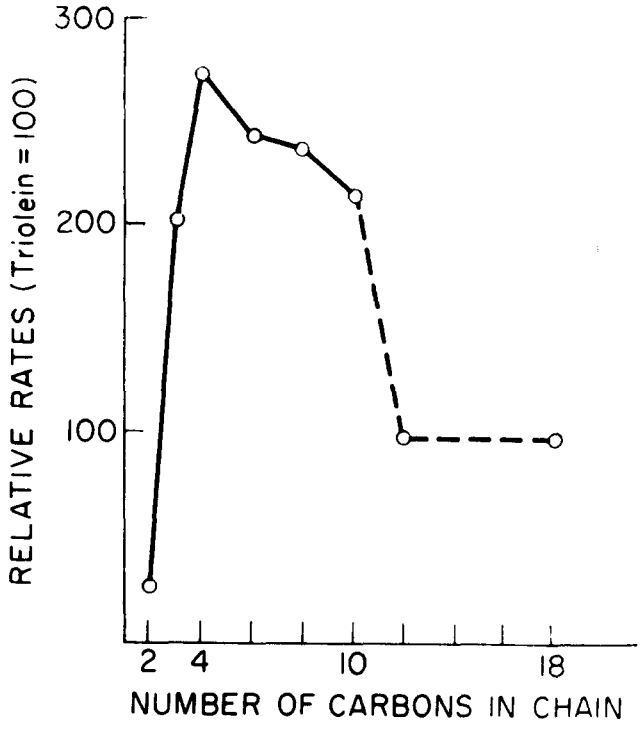

FIG. 4. Initial rate of lipolysis as a function of chain length. Case of triglycerides containing 3 identical chains (28). Optimal conditions giving maximal rates have been used for each substrate. The rates are expressed as percentage of the rate obtained when the same amount of lipase is acting on a triolein emulsion under optimal conditions. See text for the significance of the solid and dotted parts of the curve.

liberates shorter-chain fatty acids, and the specificity of intestinal acyl CoA synthetase, which activates shorter chain fatty acids very slowly (33).

Branching of the aliphatic chain in the neighborhood of the carboxyl group seems to hinder lipase action (34, 35). 2,2-Dimethyl stearic acid given orally to rats and incorporated into lymph triglycerides is resistant to subsequent hydrolysis by lipase in the presence of bile salts.

No detailed investigation has been published so far on the hydrolysis rates of positional or *cis-trans* isomers of unsaturated fatty acids. However, it has been postulated that autoxidation of the chains slows down their hydrolysis (36).

(b) *The Alcohol Moiety.* The specificity of lipase toward the alcohol moiety of its ester substrates has not been investigated in much detail. The data quoted by Ammon (1) are not conclusive because the enzyme preparations used are grossly impure and the experimental conditions during the assays are not given. An 80% hydrolysis of propyleneglycol distearate by a pancreatic preparation named "steapsin" has been recently observed (37). The same ester is readily hydrolyzed in the intestine of the rat. Fatty acid esters of sucrose (38) are also hydrolyzed by a crude preparation of pancreas.

More precise information has been obtained with fatty acyl esters of monoalcohols. The maximal rate at which these esters are hydrolyzed by purified lipase appears to be much lower than in the case of triglycerides. Thus, Fig. 2 shows that the first ester bond of triacetin is split by purified lipase 10 times as quickly as the methyl butyrate. The ratio becomes still larger when the fatty acid is the same in both substrates. It is 25 when triolein is compared to methyl oleate (6) and 50 when tributyrin is compared to ethyl butyrate (16).

Furthermore, it is known that considerable amounts of di- and monoglycerides accumulate during in vitro and in vivo hydrolysis of triglycerides. This suggests that the hydrolysis rate decreases in the order: triglycerides > diglycerides > monoglycerides. The decrease is partly explained by the positional specificity of lipase, which finds two outer chains in triglycerides, only one in 1,2-diglycerides, and none in 2-monoglycerides (see later). But some experiments show that, even when the preparations used contain mostly the isomers with outer chains (1,3-diglycerides and 1-monoglycerides), the rate is definitely lower for partial glycerides (16) than it is for the corresponding triglycerides (tripropionin, 100; dipropionin, 64; monopropionin, 22; tributyrin, 100; monobutyrin, 54).

A number of other esters have been employed for lipase assays. It is difficult to decide at the present time whether they are substrates for lipase or for "esterase." The Tweens (39) are not likely to be substrates for lipase, since an enzyme, active against this class of compounds and inactive against long-chain triglycerides, has been isolated from pancreas (40) and adipose tissue (41). On the other hand, phenolic esters such as *p*-nitrophenyl and *β*-naphthyl esters, have been recommended and often used for testing lipase activity in various tissues or biological fluids (42). The main advantage of these so-called "chromogenic" substrates is to permit a colorimetric determination of the hydrolytic process either directly or after addition of a suitable reagent. The high sensitivity of colorimetry may compensate for the relatively low rate at which this class of compounds is attacked.

The data in Fig. 3 suggest that the requirements of pancreatic lipase concerning the physical state of the substrate are the same for phenolic and carboxylic esters. *p*-Nitrophenyl esters, and probably also *β*-naphthyl esters, are suitable substrates provided they are employed in an heterogeneous system and are liquid at the temperature of the experiment. *p*-Nitrophenyl acetate is, for instance, hydrolyzed by "esterase" when it is dissolved in water and is not hydrolyzed by lipase in supersaturated solutions because of its high melting point (24). In contrast, emulsions of *p*-nitrophenyl laurate, which is liquid at 37°, are hydrolyzed by lipase. This last observation gives a new explanation to the earlier claim, according to which esterases split esters of shorter-chain fatty acids, and lipases, esters of longer-chain fatty acids.

The action of pancreatic lipase on phospholipids and plasmalogens has never been studied with pure lipase. It is probably very weak or nonexistent.

OTHER LIPASES

1. *Plasma and Tissue Lipases.* Intravenous injection of heparin induces the release into plasma of a factor able to clear in vitro the turbidity of lipemic plasma (43). Since the clearing is associated with a decrease of blood triglycerides and an increase of free fatty acids, it has been assumed that the clearing factor is a lipase acting on the triglyceride moiety of chylomicrons or low-density plasma lipoproteins. This factor is sometimes called clearing factor lipase (44).

On the other hand, ammonia extracts of acetone-dried powders of adipose and heart tissue catalyze in vitro hydrolysis of triglycerides in chylomicrons and serum lipoproteins (45, 46). These extracts are not able to act on commercial oil emulsions without prior

"activation" of the latter by serum or some fractions of serum proteins. The corresponding enzyme has been named lipoprotein lipase (47).

Lipoprotein lipase and plasma clearing factor lipase are released by heparin from intact tissues. Both are inhibited by protamine sulfate, sodium pyrophosphate, and high concentrations of sodium chloride (46, 48–50). This common behavior may suggest an identity, the enzyme being called lipoprotein lipase as long as it remains in the tissues, and clearing factor lipase when it is in the plasma. But this identity has not yet been demonstrated in a direct way. The almost immediate release of clearing factor lipase into plasma after in vivo heparin injections may suggest that at least some of the activity is localized inside the walls of the blood vessels (44, 51, 52).

Moreover, it is not easy to differentiate pancreatic lipase and lipoprotein lipase on the basis of their respective substrate specificities. Clearly, pancreatic lipase is able to split nonactivated triglyceride emulsions and even triglycerides merely shaken with a buffer in the absence of any added emulsifier. Crude preparations of pancreatic lipase are also able to hydrolyze chylomicrons, a typical substrate for lipoprotein lipase, but the initial rate of this hydrolysis is very low at the beginning and it increases with time until relatively high values are attained. It has been postulated (47, 53) that pancreatic lipase itself cannot split intact chylomicrons and that some unknown compound, or compounds, must be destroyed in chylomicrons by other enzymes of the crude pancreatic preparations before triglyceride hydrolysis by lipase takes place normally. As a matter of fact, the behavior of pure pancreatic lipase toward chylomicrons is still unknown. It has been found in our laboratory that purified preparations of pancreatic lipase do not hydrolyze chylomicrons at a slower rate than does crude lipase (54).

At least two difficulties are encountered with lipoprotein lipase. The first is to decide whether or not prior incubation of oil emulsions with serum is an absolute requirement for the enzyme. This requirement is suggested by a number of experiments. However, preparations obtained from chicken adipose tissue (55) have been reported to sometimes hydrolyze non-"activated," concentrated emulsions at a quite noticeable rate. It could be assumed in this case that the crude enzyme contains the activator. Other experiments have been performed recently (56) with rat adipose tissue homogenates. When the homogenate is used at once, the need for a prior incubation of the substrate emulsion with serum appears clearly. But the effect is much weaker when the homogenate has been maintained several hours in phosphate buffer at pH 7.4.

The second, and perhaps more serious, difficulty is that the nature of the process going on during incubation of the oil emulsion with serum is unknown. The name lipoprotein lipase cannot be considered as entirely correct as long as it is not demonstrable that incubation promotes some kind of association between proteins and emulsified triglycerides, and that the enzyme specifically acts on these aggregates.

Finally, it should be pointed out that proteins activate, not only lipoprotein lipase, but also other enzymes interacting with lipids in the cells, such as monoglyceride transacylase (57) and diglyceride transacylase (58) of intestinal mucosa. Therefore, it appears likely that several enzymes of lipid metabolism at the cellular level require a certain "mode of presentation" of their insoluble substrates. But this mode cannot, at present, be precisely described.

Other lipolytic activities have been reported to exist in adipose tissue. One of them, called adipose tissue lipase (59), has been partly purified and found to hydrolyze a number of glycerides, but not triacetin and triolein. Ammonia extracts of rat adipose tissue contain, not only a lipoprotein lipase, but also a "tributyrinase" (60). In contrast with the first, this latter activity is stable and it is inhibited by 0.1 M sodium fluoride. A lipolytic activity different from lipoprotein lipase has also been found in rat heart and adipose tissue (61). The "Tween-hydrolyzing lipase" extracted from rat adipose tissue (41), which attacks Tween 20 but is inactive against the other Tweens, commercial oil emulsions, and long-chain triglycerides, is probably an esterase rather than a lipase.

2. *Intestinal Lipase.* A few years ago, it was still undecided whether alimentary triglycerides were attacked before absorption by a single enzyme, namely pancreatic lipase, or also by a gastric lipase and an intestinal lipase. Gastric and intestinal extracts were known to possess lipolytic activity. But the difficulty was that this activity could possibly have been due to the very plentiful pancreatic enzyme, either regurgitating through the pylorus, or impregnating intestinal cells during its normal intraluminar travel. Until 1960, the single convincing argument in favor of the existence of an intestinal lipase was that lipolytic activity was found in the intestine of pancreatectomized dogs (62).

The problem of intestinal lipase has now been investigated in several laboratories. It has been shown in the first place (63) that intestinal mucosa homogenates prepared from various species are able to liberate fatty acids from olive oil emulsions and that a good

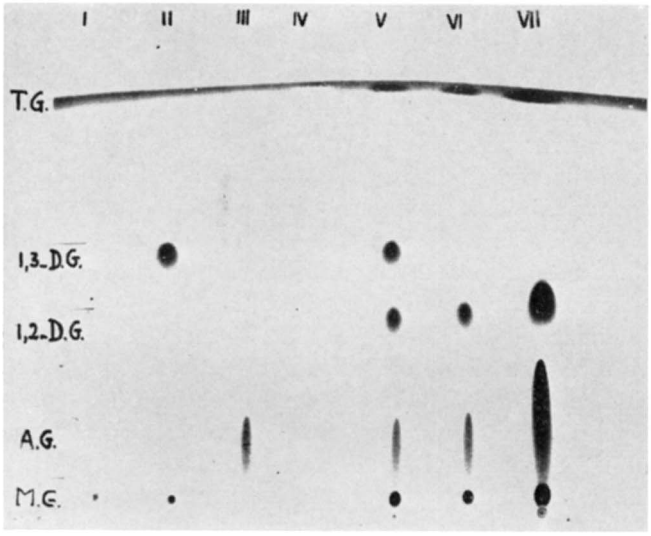

FIG. 5. Thin-layer chromatography of the lipid mixture formed during in vitro lipolysis. The technique of Privett and Blank (100) was used with the solvent system hexane ether 60:40 (v/v). Left to right: 1-monoolein (I), 1-monoolein + 1,3-diolein (II), oleic acid (III), triolein (IV), the lipid mixture obtained after lipolysis + 1,3-diolein (V), the same mixture as in V but without adding 1,3-diolein (VI), and finally an amount of lipolysis mixture twice as great as in VI (VII). TG, triglycerides; DG, diglycerides; AG, free fatty acids; MG, monoglycerides.

part of this activity is localized in the mitochondrial and microsomal fractions. This intracellular localization, as well as the absence of any accumulation of activity in the brush border of the mucosa (64), strongly suggests that this intestinal lipase is distinct from pancreatic lipase. A second argument is founded upon a comparison between the chemical specificities of both enzymes. In contrast to pancreatic lipase, the intestinal enzyme has been reported (63) to hydrolyze triolein, 1,3-diolein, and 1-monoolein at the same rate, and to display a very low activity against long-chain fatty acyl esters of β-naphthol.

However, it has been repeatedly pointed out by other workers (64–67) that the intestinal enzyme does not hydrolyze tri- and diglycerides, but exclusively 1- and 2-monoglycerides. This specificity was demonstrated by radioactive and titrimetric techniques. It appears to justify the name monoglyceride lipase for the enzyme concerned. It is not yet known whether intestinal mucosa contains two enzymes, one for triglycerides and the other for monoglycerides, or whether the different specificities found in different laboratories arise once more from a different "presentation" of the insoluble substrates. Anyway, monoglyceride lipase appears to be a true lipase, since its best substrate, monocaprin, is sparingly soluble in water. Its biological significance is discussed later.

3. *Milk Lipases.* Our knowledge concerning milk lipases is still limited. An enzyme, called milk lipase, has been found in skimmed milk. It is probably a true lipase for it hydrolyzes long-chain, water-insoluble triglycerides (68–70). A "B-esterase concentrate" has been prepared from cow's milk by adsorption on magnesium hydroxide (71). Its activity was tested on tributyrin during the purification. But it also catalyzes hydrolysis of long-chain triglycerides with the same positional specificity as milk lipase (72). In contrast to pancreatic lipase, milk lipase does not split shorter chains at a higher rate than longer ones when both types of chains occupy equivalent positions (positions 1 and 3) in a triglyceride molecule (73).

In addition, another lipolytic activity has been reported in skim milk and cream (74). This activity is similar to lipoprotein lipase in that it is highest on chylomicrons and emulsions incubated with serum. A given volume of skim milk hydrolyzes such incubated emulsions five times as quickly as tributyrin, the best substrate of B-esterase. It has been suggested that the enzyme may play a role in the uptake of plasma triglycerides by mammary gland cells (74).

New information has been gained in the recent years about ricinus lipase (75).

III. POSITIONAL SPECIFICITY

PANCREATIC LIPASE

1. *Experimental Proofs.* (a) *General case.* A tetramolecular reaction with the direct conversion of a triglyceride into a glycerol molecule and three fatty acids is very unlikely. It is much more probable that triglyceride hydrolysis occurs in three consecutive steps forming diglycerides, monoglycerides, and glycerol. A series of investigations has demonstrated that large amounts of di- and monoglycerides actually accumulate during in vitro lipolysis by pancreatic lipase (76–80) and in the intestine during in vivo digestion of dietary triglycerides (81–85). It is indeed difficult to go beyond the monoglyceride stage during in vitro lipolysis (86), unless very high amounts of enzyme and bile salts are used (87).

Conflicting information is found in the early literature about the possibility that the position of the chains within a triglyceride molecule influences its hydrolysis by pancreatic lipase (88–92). Nevertheless, it was reported several years ago (93) that a crude preparation of the enzyme splits off only one chain of tripropionin and hydrolyzes 1,2-dipropionin much more slowly than the 1,3-isomer. Both facts were interpreted as showing that tripropionin degradation by

lipase proceeded via 1,2-dipropionin, and, consequently, that hydrolysis of triglyceride outer chains (positions 1 and 3, or α and α') occurred at a higher rate than hydrolysis of the inner chain (position 2 or β). However, it was found later (94) by the same authors that, although the splitting of the inner chain was still comparatively slow, water-insoluble trilaurin and the two isomers of di- and monolaurin were completely hydrolyzed by crude lipase under the experimental conditions employed.

A step forward was made when the diolein formed by lipolysis of triolein in vitro was isolated and found to behave like the 1,2-isomer when chromic acid oxidation was performed (95). Moreover, when a mixture of triolein and palmitic-C^{14} or oleic-C^{14} acid was incubated with rat pancreatic juice, exchange between free and esterified fatty acid chains mostly occurred on the outer positions (87). This latter observation suggested a low reactivity of the inner chain.

The first direct argument in favor of a positional specificity of pancreatic lipase was obtained by in vivo experiments (83). Monoglycerides present in rat intestine were isolated by countercurrent distribution and found to contain large proportions of the 2-isomer. Moreover, when rats were given synthetic 2-oleyl dipalmitin (POP), the iodine value of the monoglyceride fraction of intestinal lipids was nearly equal to that of monoolein, whereas it was very low for the free fatty acid fraction.

Since then, detailed investigations (30, 31, 96, 97) have been carried out in vitro with more refined techniques. The four possible glycerides of palmitic and oleic acids (two symmetrical compounds: 2-oleyl dipalmitin [POP] and 2-palmityl diolein [OPO]; and two unsymmetrical compounds: 1,2-dipalmityl 3-olein [PPO] and 1,2-dioleyl 3-palmitin [OOP]) were synthesized and subjected to hydrolysis by lipase until a predetermined proportion of acids was liberated. The free acids were separated on Amberlite IRA-400, and the amounts of 1- and 2-monoglycerides were estimated by periodic acid oxidation before and after perchloric acid isomerization (98). The tri-, di-, and monoglycerides were fractionated by countercurrent distribution or chromatography on silicic acid columns. The composition of the fatty acids in all glyceride fractions and the composition of the free fatty acids were calculated from the iodine value or directly determined by gas chromatography. All results were consistent with the assumption that most free acids came from the outer positions of the synthetic triglycerides and that most chains found in the monoglyceride fraction were inner chains.

Since randomly distributed palmityl and oleyl chains are split at similar rates (see above), the conclusion seems inescapable that lipase splits off outer chains of triglycerides much more rapidly than inner chains. Furthermore, the acids liberated from unsymmetrical glycerides (PPO and OOP) contain nearly equal amounts of palmitic and oleic acids. This suggests that the two outer positions of the glyceride molecule are equivalent as far as lipolysis is concerned.

Now, the question remains whether the inner chains of the triglyceride molecules are slowly split by lipase, or not at all. Figure 5 shows that the diglycerides formed during an in vitro lipolysis of triglycerides are almost entirely of the 1,2-configuration. The first observations suggest that the enzyme exclusively splits the outer chains. But it has been repeatedly pointed out that the monoglyceride fraction obtained after 1 hr of lipolysis at pH 8.0 contains as much as 20–30% 1-isomers and only 70–80% 2-isomers. (97).

The presence of 1-monoglycerides at the end of the lipolysis process does not prove a lack of specificity of lipase. These compounds may arise by isomerization of preformed 1,2-diglycerides or 2-monoglycerides. Indeed, it has been recently shown (101) that 2-monoglycerides are very unstable when mixed with water at alkaline pH. When synthetic pure 2-monoolein, for instance, is suspended in water at 37° and pH 8.0, isomerization occurs so rapidly that equilibrium (88% of the 1-isomer for 12% of the 2-isomer) is reached within a few minutes. Thus, the surprising fact is not to find 20–30% 1-monoglycerides after in vitro incubation or intestinal digestion, but that 70–80% of 2-monoglycerides has escaped isomerization.

A plausible explanation of this fact has been found in the stabilization effect of free fatty acids. In the presence of free acids, isomerization of 2-monoglycerides is slowed down and it stops when about 20–30% of 1-isomers is formed (99). Hence isomerization of 2-monoglycerides during lipolysis is probably slower than normal but, nevertheless, it can amply explain the formation of the observed quantity of 1-isomers at the end of the process.

The above discussion and other experimental evidence do not imply, of course, that outer chains are being exclusively liberated during lipolysis. Some inner chains may be split after isomerization to position 1 or 3. But they suggest in a rather convincing way that the positional specificity of pancreatic lipase is very strong indeed, if not absolute. The main pathway of in vitro and in vivo lipolysis is: triglycerides → 1,2-diglycerides → 2-monoglycerides. The formation of free glycerol under certain circumstances (81, 86, 87) should be chiefly attributed to the isomerization of unstable partial glycerides. 2-Monoglycerides are not hydro-

lyzed by lipase even when they are associated with bile salts in mixed micelles (23).

(b) *Special case of glycerides containing shorter chains.* The rapid hydrolysis of shorter fatty acid chains by lipase enhances the positional specificity of the enzyme when these chains are bound to outer carbons. But it is interesting to know what happens when shorter chains are bound to the inner carbon.

The lipolysis of tricaprylin under normal conditions presents two unusual features. Large amounts of free glycerol are formed, and the proportions of 1-isomer in the monoglyceride fraction may be as high as 80% (99). This means that, in contrast to longer chains, the caprylyl chain bound to the inner carbon in the tricaprylin molecule is rapidly released. A first possibility is that the known preference of lipase for shorter chains outweighs the positional specificity of the enzyme. A second possibility that has been experimentally verified (99) is that 1,2-dicaprylin and 2-monocaprylin, and more generally di- and monoglycerides having a shorter chain on the inner carbon, isomerize at an especially high rate. This characteristic behavior explains why the monoglycerides derived from 2-butyryl dipalmitin contain as much as 40% palmityl chains (102). After its migration to an outer carbon, the butyryl chain is split more rapidly than the palmityl chain and 1-monopalmitin accumulates. It should be stressed here that, when the triglyceride substrate contains shorter chains bound to the inner carbon, high proportions of originally outer chains appear in the monoglycerides and high proportions of originally inner chains appear in the free fatty acid fraction following hydrolysis.

2. *Use for the Determination of Glyceride Structure.* (a) *Natural triglycerides.* By glyceride structure is understood the mode of association of the free fatty acid chains in triglyceride molecules. The glyceride structure of natural fats is important since it exerts a strong influence on some of the physical properties of these fats and it may also give valuable information about the mechanism of triglyceride biosynthesis in plants and animals.

Our knowledge in this field until 1955 has been ably reviewed (103). Triglycerides with two or three identical chains are seldom found in nature. According to the so-called even distribution theory (104), the probability of associating identical chains in the same triglyceride molecule is always considered to be at its lowest possible level. Thus, two or three chains will begin to associate only when the proportions of this type of chain in the fat are higher than 33 or 66%, respectively.

Some difficulties, mainly encountered with animal fats, led a little later to the belief that the chains are randomly distributed (105, 106). Random distribution implies a much higher proportion of trisaturated and triunsaturated glycerides than even distribution. An evenly distributed fat containing equal amounts of palmityl and oleyl chains contains no tripalmitin or triolein. If randomly distributed, the same fat contains 12.5% of these glycerides and 37.5% of the mixed-acid glycerides dioleyl palmitin and dipalmityl olein. In each group of mixed-acid glycerides, there will be $^1/_3$ of the symmetrical species (2-oleyl dipalmitin or 2-palmityl diolein) and $^2/_3$ of the unsymmetrical one (1,2-dipalmityl 3-olein or 1,2-dioleyl 3-palmitin).

However, the sometimes large modifications brought about by interesterification in the physical properties of a number of natural fats soon showed that the theory of random distribution was incorrect. Then it was postulated that the random distribution was merely "partial" (107) or "restricted" by the obligation for the triglycerides to remain liquid *in situ* (108). Indeed, this latter kind of distribution would have required a rather complicated mechanism for the regulation of triglyceride biosynthesis and for the transfer of the necessary information.

All these uncertainties undoubtedly arose from a nearly complete lack of suitable techniques for the systematic fractionation of natural fats into their elementary triglycerides. When pure triglycerides could be isolated, it was obvious that the positions occupied by the chains were controlled to some extent by their chemical nature. For instance, all samples of distearyl olein crystallized from vegetable fats were found to have the oleyl chain bound to the inner carbon. (109). Conversely, palmityl chains were reported to be bound to inner carbons in a series of glycerides isolated from pig fat (109, 110).

The positional specificity of pancreatic lipase offers an almost ideal means for investigating the distribution of the fatty chains in triglycerides (30, 97, 111–117). If the nature of outer chains is determined, it is probably advisable to stop the hydrolysis as early as possible. As stated above, the 2-monoglycerides and also the 1,2-diglycerides formed by lipase are very unstable in aqueous suspensions at alkaline pH. This isomerization induces the transfer of originally inner chains to outer positions and thus promotes their hydrolysis. Isomerization is a less serious problem and fewer inner chains are found in the pure fatty acid fraction when triglycerides are treated by lipase during a short time.

On the other hand, the presence of outer chains in the monoglyceride fractions produced by hydrolysis is much less likely, at least in the general case of long-chain glycerides. Thus, when the nature of inner

chains is being investigated, it is probably permissible to hydrolyze the fat to an extent of about 60%, which corresponds to a maximal production of monoglycerides (86). Prior destruction of 1-isomers in the monoglyceride fraction by periodic acid oxidation (113) is probably unnecessary. As pointed out earlier, these isomers are formed by isomerization of 2-monoglycerides rather than by the direct action of lipase. Thus, they mostly contain originally inner chains. It has been experimentally verified in our laboratory that the fatty acid compositions of the 1-monoglycerides and 2-monoglycerides formed by hydrolysis are similar.

It has been recently postulated (117) that the positional specificity of lipase is improved by purification. This would imply that crude preparations from pancreas contain either a second triglyceride-splitting enzyme with a different specificity or an "isomerase" catalyzing intramolecular transfers of fatty chains. Both possibilities are unlikely. Exchanges induced by lipase between free and esterified chains are not considered here, since they occur mostly on outer positions (87) from which the free fatty acids are derived. Thus, the important point seems to use fully active enzyme preparations. When activity is low, hydrolysis takes longer and the danger of unwanted isomerization increases. It is certainly advisable to test by known techniques the activity of the enzyme used. So far, better results have not been obtained with a purified preparation than with a crude one containing the same number of lipase units.

The general conclusions reached by all investigators using the lipase technique is that the structure of natural triglycerides is never random. Two cases should be considered. In mixed-acid glycerides of vegetable fats, position 2 is entirely, or almost entirely, occupied by unsaturated fatty acid chains, even when the proportions of these chains in the fat do not exceed 33%. Consequently, all, or almost all, saturated chains are bound to outer carbons together with the remaining unsaturated chains. This means that if saturated and unsaturated chains are designated by the letters S and U, disaturated glycerides of vegetable origin are chiefly of the SUS form, whereas the monosaturated glycerides are largely in the UUS form. No obvious difference can be noted between palmityl and stearyl chains in the saturated group. For the unsaturated group, oleyl and linoleyl chains are, in most cases, preferentially bound to inner carbons. But the behavior of both chains is not always identical. In cottonseed and peanut oils, for instance, oleyl chains are evenly distributed on inner and outer positions, whereas linoleyl chains are mostly on inner positions. The reverse situation exists in soya bean oil. Finally, chains containing more than 18 carbons are bound to outer carbons (114) regardless of saturation or unsaturation.

The distribution of saturated and unsaturated chains in animal fats is not so straightforward. Both types of chains are found on outer and inner positions. Nevertheless, a predominance of saturated chains on outer carbons and of unsaturated chains on inner carbons is also noted in these fats, with the unique exception of the depot fat of the pig and animals belonging to the same family, where the reverse type of distribution is found. In this fat, palmityl chains are mainly bound to inner carbons whereas stearyl chains are mainly found with unsaturated chains on outer positions.

In the case of milk fat and other fats containing shorter chains, the results given by the lipase technique are probably erroneous. As stated above, experiments performed with synthetic triglycerides suggest that shorter chains originally bound to the inner position may appear in the free fatty acid fraction whereas longer chains originally bound to the outer carbons appear in the monoglyceride fraction. For this reason, the observation that butyric acid is exclusively bound to outer carbons in butter fat triglycerides (118) should be considered with caution. The position of other shorter chains in milk fat (119) is also dubious.

(b) *Chylomicron triglycerides.* The extent to which alimentary triglycerides are hydrolyzed in the intestinal lumen before penetrating mucosal cells has been the object of an active controversy for more than a century. After oral administration of isotopically labeled triglycerides to rats, it is found that a part of the labeled glycerol backbone is recovered in the chylomicrons (120, 121). If the assumption is made that free glycerol, or at least glycerol liberated from dietary fats in the lumen, cannot be utilized for triglyceride resynthesis in the mucosa, this observation shows that a part of triglyceride glycerol can cross the intestinal wall in an esterified form and, consequently, that complete intraluminar hydrolysis of triglycerides is not compulsory for absorption. From a study of the absorption of a mixture of doubly-labeled unsaturated glycerides and of unlabeled saturated glycerides, it has been calculated that 25–45% of ingested fats are completely hydrolyzed in the lumen and that the remainder is absorbed as monoglyceride (121). In order to eliminate the possibility of exchanges between the fatty acid chains of the glycerides in the mixture, lipase-resistant 2,2-dimethylstearyl glycerides were used (85). It was found that 40% of the chains are absorbed in the free state and 60% in the form of partial glycerides. The possibility that monoglycerides penetrate the mucosa is in good agreement with the new theory (122) according

TABLE 1. LIST OF ENZYMATIC ACTIVITIES ATTRIBUTED TO LIPASES

Origin	Substrates Used	References
(a) Animal		
Brain	Tweens 20, 40, 60, and 80; tributyrin	148
Rana esculenta (larva and embryo)	Tributyrin emulsion	149
Central nervous system	Tween 60	150
Skeletal muscles of pigeon, frog, and fowl	Tributyrin, triacetin, olive oil, castor oil	151
Heart muscle of various animals	Tributyrin emulsion	152
Pigeon muscle	Tween 80	153
Adipose tissue of vertebrates	Tributyrin	154
Adipose tissue	Various triglycerides, does not split triacetin or triolein	59
Rat adipose tissue	Tween 20 (not other Tweens); hydrolyzes short-chain (C_1-C_8), but not long-chain triglycerides	41
Housefly	Acts on various triglycerides but not on Tweens 60 and 80 or ethyl esters.	155
Artemia salina	Tributyrin emulsions	156
Bombyx mori	Tributyrin emulsions	157
Lingcod muscle	Triacetin, tripropionin, tributyrin. The hydrolysis of methyl and ethyl acetates is very slow, and still slower for tricaprylin and olive oil	158
(b) Vegetable		
Wheat germ	Triacetin, tripropionin, short-chain esters	159
Various seeds	Tributyrin (and olive oil for the more active preparations)	160–162
Oat seeds	Tributyrin	143
Gramineae seeds	Methyl oleate	163
Rye	Triacetin, tributyrin, various methyl esters	164
Germinated rapeseed	Triacetin, tributyrin, long-chain triglycerides	165
Poppy, flax, and sunflower seeds	Various oils; ethyl and butyl acetates	166
Peas	Pea lipids, tributyrin	167
(c) Microorganisms		
Fungal	Tributyrin	168
Mycobacterium ranae	Tributyrin	169
Aspergillus	Emulsified peanut oil	170
Pseudomonas	Long-chain triglycerides	171–173
Bacteria (seven species)	Olive oil, tributyrin, and ethyl esters	174

to which mucosal cells absorb a micellar dispersion of monoglycerides, free fatty acids, and bile salts.

The same problem can also be investigated by determining with the lipase technique the nature of inner chains of chylomicron triglycerides (123). It has been found that these inner chains are quite different when the animals are fed with triglycerides or with the fatty acid mixture obtained therefrom by saponification. In the first case, the inner chains of fed triglycerides are recovered to an extent of about 70% on the same position in the chylomicron triglycerides. In the second case, the distribution of the fatty chains in chylomicron triglycerides is nearly random, except for stearyl chains, which are mainly bound to outer carbons (123). These results have been essentially confirmed by an independent study performed with synthetic triglycerides or mixtures of free fatty acids containing radioactive palmitic acid (124). Thus, it is now fairly well established that intraluminar fat digestion is incomplete. Ingested triglycerides are mainly converted by pancreatic lipase into 2-monoglycerides and free fatty acids. Both types of compounds penetrate the mucosa and are utilized for the resynthesis of chylomicron triglycerides. Intestinal mucosa is able to synthesize chylomicron triglycerides, not only by the classical pathway (125) starting from α-glycerophosphate, but also by converting intraluminar 2-monoglycerides into 1,2-diglycerides (57, 58).

(c) *Structure of phosphatides.* A similar technique has been used for investigating the structure of natural glycerophosphatides (126). A purified egg phosphatidyl choline preparation was treated by a phosphatidase D from *Cl. perfringens*, which removes the phosphoryl choline group. The stability of the resulting 1,2-diglycerides was improved by acylation of the free hydroxyl group, performed by myristic acid, which is not present in the original preparation. The triglyceride obtained was treated for a short time (15 min) by pancreatic lipase, which liberated only myristic, palmitic, and stearic acids. Thus, it was concluded that the outer chain of egg phosphatidyl cholines is saturated and that the inner chain is unsaturated. It is very interesting to note that this distribution pattern of fatty chains in glycerophosphatides is similar to the pattern found in most natural triglycerides. According to accepted theories (125), triglyceride and phosphatide biosynthesis proceed by a common pathway from α-glycerophosphate up to the 1,2-diglyceride stage. The enzyme involved in the attachment of the inner chain close to the phosphoryl radical is likely to have a marked specificity for unsaturated chains.

The distribution of saturated and unsaturated chains in glycerophosphatides also raises the question of the positional specificity of phosphatidase A from snake venom, which converts phosphatides into lysophosphatides by the removal of one of the two fatty acids. Earlier investigators in this field had claimed either that the enzyme released the outer chain (127–129) or that the specificity of the enzyme was controlled by the chemical nature of the chain as well as by its position (130, 131). Further study of the action of phospholipase A on a series of synthetic mixed-acid phosphatides (132–135) proved later that the enzyme exclusively splits the inner unsaturated chain of phosphatidyl cholines and forms a saturated lyso derivative. Thus, the positional specificity of phospholipase A is exactly the reverse of the specificity of pancreatic lipase.

(d) *Structure of synthetic glycerides.* The lipase method may, finally, be used to check the purity of

synthetic mixed-acid triglycerides or partial glycerides (136, 137). Di- or monoglycerides are stabilized as above by acylation with an acid not present in the original product. The sensitivity of the technique for detecting low amounts of "wrong" isomers in synthetic products is limited by the possibility of isomerizations taking place during acylation and digestion with lipase. But it is certainly useful in conjunction with other techniques, such as thin-layer chromatography of diglycerides (100) and monoglycerides (138).

OTHER LIPASES

In contrast to pancreatic lipase, ricinus lipase seems to form low amounts of partial glycerides and high amounts of free glycerol (139). This suggests that the positional specificity of the enzyme is relatively poor. A second argument favoring the same view is given by a comparative study of cocoa butter hydrolysis by the pancreatic and the ricinus enzymes (139). In the first case, outer saturated chains are liberated. In the second, the average unsaturation of the liberated chains does not differ significantly from that of the total chains.

Despite some conflicting observations (140, 141), lipoprotein lipase also seems to hydrolyze the three chains of its substrate at similar rates (142).

Milk B esterase has been reported to have a marked specificity for outer chains (68–70). Milk lipoprotein lipase seems to hydrolyze outer chains of activated coconut oil more rapidly than the inner ones. But hydrolysis appears to be random when chylomicrons are used (74). Oat lipase has been reported to hydrolyze a single chain from tributyrin (143).

IV. STEREOCHEMICAL SPECIFICITY OF PANCREATIC LIPASE

The last question is to see whether pancreatic lipase, like most other enzymes, possesses a stereochemical specificity. A first possibility of stereoisomerism in a glyceride molecule is offered by the presence of an asymmetrical carbon in 1,2-diglycerides and 1-monoglycerides as well as in triglycerides and 1,3-diglycerides having two different outer chains. D-1,2-Diglycerides, for instance, are better substrates for phosphatide biosynthesis than their L-enantiomorphs (125). It would be interesting to know whether the first form is preferentially produced, not only by enzymatic dephosphorylation of L-1-phosphatidic acids, but also in the intestine by lipase. Therefore, D, L, and DL 1,2-dipalmityl 3-olein have been prepared by acylation of the corresponding stereoisomers of 1,2-dipalmitin. The diglycerides formed by lipase digestion have been isolated and found in no case to have any detectable rotatory power (144).

Furthermore, it is known that apparently symmetrical compounds such as citric acid and glycerol behave as if they exist in two enantiomorphic forms (145, 146). This fact can be used for checking a possible stereospecificity of lipase in two ways (147). The first one is to lipolyze D-1-(oleyl-1-C^{14})2,3-dioleylglycerol to the diglyceride stage and to compare the specific radioactivity of the diolein and oleic acid formed during the digestion. The second is to saponify the trityl derivative of the diglycerides produced by trioleyl D-glycerol-1-C^{14} digestion and to determine the specific radioactivity of the outer carbon recovered as formaldehyde during periodic oxidation of the compound obtained. No sign of stereospecificity of lipase can be detected by either technique.

V. A LIST OF ENZYMATIC ACTIVITIES ATTRIBUTED TO LIPASES

The above review has been mainly devoted to a group of enzymes hydrolyzing emulsified triglycerides. It is unfortunate that, among the large number of "lipases" quoted in the literature, the reader cannot always recognize the enzymes whose requirements clearly satisfy this unequivocal definition. Some of the enzymes concerned are listed in Table 1 with the substrates on which the corresponding activities have been measured.

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