Relative rates of hydrolysis by rat pancreatic lipase of esters of C_2–C_{18} fatty acids with C_1–C_{18} primary *n*-alcohols

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ABSTRACT The rate at which rat pancreatic lipase (glycerol-ester hydrolase, EC 3.1.1.3) hydrolyzes the esters of primary *n*-alcohols containing from 1 to 18 carbon atoms with fatty acids containing from 2 to 18 carbon atoms was determined. The speed of hydrolysis was influenced, apparently independently, by both the acyl and the alkyl chains. With respect to the fatty acid moiety, the esters of dodecanoic acid were usually split at the most rapid rate. Esters of butyric acid were the next most susceptible. In the case of the alcohol moiety, esters of heptyl alcohol were hydrolyzed most rapidly.

On the basis of the pattern of the relative rates of hydrolysis, it is proposed that the influence of the alcohol component is a result of its orienting the ester molecule at the oil/water interface. The fatty acid effect is attributed to enzyme–substrate specificity.

SUPPLEMENTARY KEY WORDS substrate specificity · orientation at oil/water interface

PANCREATIC LIPASE (glycerol-ester hydrolase, EC 3.1.1.3) has been shown to hydrolyze specifically the esters of primary alcohols (1). The specificity, if any, with respect to the fatty acid and alcohol moieties has not been established with certainty. One difficulty in establishing substrate specificity is that the enzyme functions only at an interface (2). As a consequence, the physical properties of the substrate may cause the molecule to assume a particular orientation at the interface. This alignment may or may not be optimal for the formation of the enzyme-substrate complex. The over-all rate of the reaction thus may be determined by the orientation that the substrate assumes at the interface, rather than the factors that normally are rate-controlling in a homogeneous system. Most studies have not taken this into account.

There are several reports on the rates of hydrolysis of simple triglycerides (3–9). The use of such materials in comparative studies is limited by their melting points. If the substrates used encompass triglycerides with melting points above and below the temperature of the digest, the reaction site would be a solid/liquid interface in the first instance and a liquid/liquid interface in the second. It is likely that diffusion and surface area, and possibly other important variables, would differ in these two systems. Comparing the rates obtained under such divergent conditions would not be justified. For example, the marked effect of temperature on the rate of hydrolysis when the substrate (stearate esters) has a high melting point was demonstrated in the experiments of Balls, Matlack, and Tucker (5). Triglycerides have an added disadvantage in that the products of hydrolysis, di- and monoglycerides, are themselves substrates.

Because of considerations such as these, we applied the following criteria in selecting our experimental conditions. We used esters of straight-chain fatty acids and of monohydric *n*-alcohols, thus avoiding the complications introduced by esters of polyalcohols. The concentration of substrate exceeded that of its solubility in water; thus an interface was assured. Since the solidification point of all substrates was lower than the temperature of the digest, the interface was liquid/liquid. Pancreatic juice that had been subjected to the minimum treatment necessary to inactivate the lipolytic enzymes other than lipase was used. The digestion conditions were such that diffusion would not be the rate-limiting step and factors that might influence the orientation of the substrate at the interface, when these were susceptible to control, would be the same in all experiments.

METHODS

The esters of primary *n*-alcohols containing 1–18 carbons and fatty acids containing 2–18 carbons were prepared. The fatty acids were isolated from natural fats or purchased, while the alcohols were obtained from commercial sources. The fatty acids and alcohols and the esters synthesized from them were purified by appropriate distillation, crystallization, and column chromatography. Thin-layer and gas–liquid chromatography established that the starting materials and the final products were better than 99% pure.

The lipolytic enzymes, other than lipase, in rat pancreatic juice that had been freeze-dried were inactivated by keeping a pH 9 solution of reconstituted juice at 40°C for 1 hr. The methods for obtaining, storing, and treating the pancreatic juice have been described previously (10). The activity of the enzyme preparation varied slightly from day to day. To correct for this we hydrolyzed replicate samples of methyl oleate each day. The values obtained that day for the other esters were corrected to a standard value for the methyl oleate.

The rates of hydrolysis of the esters were determined with the aid of a pH-stat. The digestion mixture consisted of 225 μmoles of substrate (some exceptions are noted later), 330 μmoles of $CaCl_2$, 7 μmoles of free oleic acid (this addition is discussed later), 17 mg of histidine (final concentration 0.002 M), 3.11 g of NaCl (final concentration 1 M), and 0.6 mg of selectively inactivated, lyophilized rat pancreatic juice in a total volume of 55 ml at pH 9.0 and at 25°C. The rate of stirring of the digestion mixture was such that a further increase in the amount of agitation did not cause an increase in the rate of enzymatic hydrolysis (11). The gas space in the flask was continuously flushed with CO_2-free, water-saturated nitrogen. If the digestion is carried out at pH 9 all of the resulting free fatty acids are titrated (11). This pH is within the broad pH optimum of this enzyme (10).

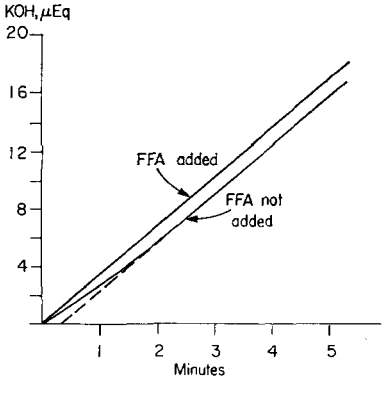

FIG. 1. Effect of added free oleic acid (FFA) on the hydrolysis by lipase of an ester composed of a short-chain alcohol and of a fatty acid of 12 or more carbon atoms. Tracings of the pH-stat recorder plots are presented.

TABLE 1 EFFECT OF ADDED FREE FATTY ACID AND CALCIUM IONS ON METHYL OLEATE HYDROLYSIS*

Additives		
Oleic Acid	Ca^{++}	Rate of Hydrolysis
7 μeq	*330 μmoles*	*μeq/min/mg enzyme*
—	—	1.8
—	—	1.6
+	—	1.8
—	+	1.7–3.1†
+	+	3.1

* Digestion conditions were as described in text except for variables noted in table.

† Rate was not constant; see text and Fig. 1.

For the majority of the substrates, the rate of the reaction was enzyme-limited under the digestion conditions described above. However, the water solubility of a few of the esters, where both the fatty acid and alcohol were short-chain, was greater than 225 μmoles per 55 ml. In these instances, the quantity of substrate was increased until the solubility limit was exceeded; thus, an interface was present. The level of ester then was raised until no further increase in the rate of hydrolysis was obtained; this level of substrate was used in determining the rate of hydrolysis. Thus all reactions were zero order with respect to substrate.

Calcium chloride, but not free oleic acid, was added to the incubation mixtures during the initial experiments in this study. We noted that the hydrolysis of an ester of a long-chain fatty acid, such as methyl oleate, showed an initial lag that lasted for 1–2 min, or until about 5 μeq of fatty acid had been released. The rate of splitting then became maximal and linear. This lag could be removed by adding to the system, before the enzyme was introduced, a small amount of oleic acid. The two types of hydrolysis curves are shown in Fig. 1; these are tracings of the plots made by the pH-stat recorder. The rates attained either initially in the presence of added oleic acid or after the first few minutes when free fatty acid was not added were the same. It was further shown that the lag period was progressively shortened as more oleic acid was added, up to a level of approximately 5 μeq. Any further addition did not affect the reaction. This effect of added oleic acid and its relationship to calcium ion is shown in Table 1.

This lag in the rate of hydrolysis in the absence of added free fatty acid was observed with esters of C_{12} or longer fatty acids. However, it was not seen with esters of the shorter-chain fatty acids; with these substrates, the rate was linear from the time the enzyme was added. The effects of added hexanoic or oleic acid or of calcium on the rate of digestion of hexyl hexanoate are presented

TABLE 2 EFFECT OF ADDED FREE FATTY ACID AND CALCIUM IONS ON HEXYL HEXANOATE HYDROLYSIS*

Additives			Rate of Hydrolysis
Hexanoic Acid	Oleic Acid	Ca^{++}	
15 μeq	*7 μeq*	*330 μmoles*	*μeq/min/mg enzyme*
—	—	—	1.5
—	—	+	1.4
+	—	—	1.5
+	—	+	1.5
—	+	—	2.7
—	+	+	2.9
+	+	+	3.0

* Digestion conditions were as described in text except for variables noted in table.

in Table 2. The introduction of either hexanoic acid or calcium, or both, did not change the rate of hydrolysis, whereas oleic acid approximately doubled the rate. If calcium was added, as well as oleic acid, there was a small further increase in hydrolysis.

In these studies we were interested in determining the relative rates of hydrolysis of esters of both short- and long-chain fatty acids. The accelerating activity of the long-chain fatty acids could not be avoided, because in the course of the hydrolysis of the esters of such acids, the fatty acids released have an accelerating effect. As a consequence 7 μeq of free oleic acid was added to all incubation mixtures. We are inclined to attribute this effect of free, long-chain acids and calcium to substrate orientation.

The values for the rates of hydrolysis of the esters are for the first few minutes after addition of the enzyme. After making a large number of measurements, we judge that a 10% difference between two rates is significant.

TABLE 3 RATE OF HYDROLYSIS OF ESTERS OF FATTY ACIDS WITH PRIMARY *n*-ALCOHOLS BY PANCREATIC LIPASE

	Fatty Acid									
Alcohol	3	4	5	6	7	8	10	12	16	18:1
	μeq/min/mg enzyme									
1	C	C	1.6	2.0	2.4	2.8	3.1	3.4	S	3.2
2	C	1.8	1.2	1.2	1.4	1.4	1.4	1.7	1.7	1.6
3	2.6	3.5	1.4	1.8	2.6	2.6	2.2	4.0	2.2	2.3
4	1.6	2.9	1.2	1.3	2.1	2.6	2.6	3.0	2.5	2.8
5	1.7	2.4	1.4	1.3	1.3	2.1	2.6	2.5	2.0	2.0
6	3.5	6.5	3.0	2.1	4.3	4.8	5.2	5.9	5.0	4.7
7	7.0	6.1	3.2	4.4	4.9	6.8	6.7	8.2	6.1	6.1
8	3.6	5.2	2.5	2.9	5.0	5.5	6.1	6.9	4.9	6.0
12	4.2	4.8	3.1	3.1	4.1	4.1	5.4	S	S	5.4
16	1.8	4.2	1.6	2.6	1.9	4.3	—	S	S	3.3
18:1	2.0	3.6	1.9	2.1	2.9	2.9	3.6	3.3	S	3.7

C, chemical hydrolysis; S, solid. Fatty acids and alcohols are designated by chain length. 18:1, oleic acid and octadec-9-enol, respectively.

RESULTS

The rates of hydrolysis of the various esters are given in Table 3. Values for the acetate esters are not listed because these were so low in all instances that reliable figures could not be obtained. For most of the alcohols, the maximum rate of hydrolysis was realized when the esterified fatty acid was butanoic or dodecanoic. Another characteristic of the series was the slow rate of splitting when the fatty acid was pentanoic or hexanoic. A pattern, although a different one, can be related to the esterifying alcohol. As the chain length of the alcohol increased, the rates of hydrolysis showed a minimum at pentanol and a maximum at heptanol. Esters of ethanol, like those of acetate, were hydrolyzed very slowly.

The rates of hydrolysis of the various fatty acid esters of each alcohol were expressed as a percentage of the rate for the ester of the alcohol that showed the maximum rate of hydrolysis. For example, each value in the row in Table 3 for the octyl esters was divided by 6.9 (octyl dodecanoate), the maximum rate observed in this series. The pattern of percentages of all of the rows (alcohols) was similar, which must mean that the relative rates of hydrolysis of esters are determined by the fatty acid independently of the alcohol component. Similarly, the hydrolysis rate for each ester of a given fatty acid was expressed as a percentage of the rate of the one that was hydrolyzed most rapidly. Thus in the case of the octanoate esters each value in this column in Table 3 was divided by 6.8 (heptyl octanoate). A pattern of percentages, which was similar for all of the columns (fatty acids), was obtained. Thus the relative rates of hydrolysis are also determined by the alcohol independently of the fatty acid component.

TABLE 4 EFFECT OF FATTY ACID AND ALCOHOL MOIETIES ON THE RELATIVE RATES OF HYDROLYSIS*

Chain Length	Alcohol	Fatty Acid
1	46.3 ± 1.6†	—
2	26.3 ± 3.2	<15
3	42.2 ± 2.1	63.5 ± 4.4
4	37.7 ± 1.9	90.3 ± 3.2
5	32.7 ± 1.9	47.3 ± 3.2
6	75.7 ± 5.3	50.6 ± 3.2
7	99.2 ± 0.1	68.6 ± 2.8
8	81.1 ± 4.8	78.3 ± 1.9
10	—	85.8 ± 4.2
12	76.6 ± 4.0	96.7 ± 1.5
16	50.7 ± 5.4	76.0 ± 4.5
18:1	49.6 ± 3.5	84.4 ± 4.4

* The rates of hydrolysis (Table 3) of the various esters of a given alcohol were expressed as a percentage of the rate for the ester of that alcohol series showing the maximum rate of hydrolysis. The average of these percentage values for each alcohol is presented. The data for each of the fatty acid series were treated similarly.

† SEM = $[\{\Sigma(x - \bar{x})^2\}/n(n-1)]^{1/2}$. n can be deduced from Table 3.

The percentage values for each set of alcohols and for each set of fatty acids were averaged. These average values are shown in Table 4. Regardless of the fatty acid moiety, the rate of hydrolysis of these esters decreased as the alcohol chain length increased from methanol to pentanol. The ethanol esters were an exception to this pattern. Further chain elongation resulted in an increase in the rate of hydrolysis, the maximum being obtained with the heptanol esters. Similarly the effect of the fatty acid portion of the ester is independent of the alcohol with which it is esterified. For this component, there is a progressive increase in the rate of hydrolysis as the chain length of the fatty acid increases from 5 to 12 carbon atoms. The esters of the C_3 and C_4 fatty acids do not conform to this pattern.

In the above treatment of the data, we considered the role of the alcohol and fatty acid moieties separately using relative rates within classes. The rate for any given ester will, of course, be dependent on both components. From the patterns in Table 4, the maximum rate of hydrolysis should be obtained, and indeed was obtained, when heptyl dodecanoate was the substrate. An examination of the data in Table 3 does not reveal any relationship that involves an interaction of the fatty acid and the alcohol; the rates of hydrolysis appear to be simply a reflection of the separate effects of the alcohol and fatty acid moieties.

DISCUSSION

The different rates of hydrolysis among these esters could be due to (*a*) the inherent reactivity of the ester group, (*b*) the orientation of the molecule at the oil/water interface, which would influence the association of the enzyme with the substrate, or (*c*) enzyme–substrate specificity. It is unlikely that reactivity of the ester group is the determining factor, since the ease of chemical hydrolysis in a homogeneous system decreases as the chain length of the acyl or alkyl group is increased up from 1 to 4 carbon atoms. Any further increase in chain length has no effect on the rate of chemical hydrolysis (12). This was not the pattern observed in our experiments. Consequently the rate of hydrolysis is determined either by the interfacial orientation of the substrate, by enzyme–substrate specificity, or by both.

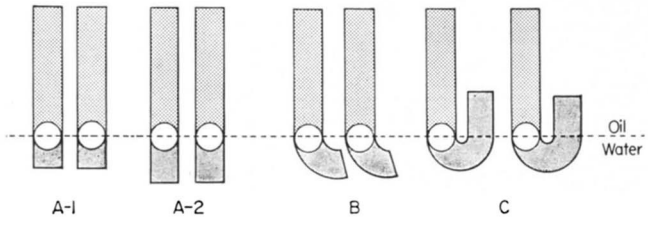

FIG. 2. Proposed orientation of esters at an oil/water interface. Open circle represents the ester linkage; bars, the acyl and alkyl chains.

At present there is no adequate physical method for determining the orientation of esters at an oil/water interface. Because the ester linkage is hydrophilic, it is reasonable to assume that this portion of the molecule will be directed to the interface. This tendency will be reinforced by the hydrophobic nature of the alkyl and acyl chains.

The investigation of monomolecular films of esters at an air/water interface is easier. Thus, Adam (13) has determined the force–area relationship of several of the esters used in the present study. From these he proposed various types of structures that an ester could assume at an air/water interface. A slightly modified version of these is depicted in Fig. 2. Although he dealt with both expanded and compressed films, only the expanded film is considered here, because it probably resembles the oil/water interface. Structure *A* is realized when one hydrocarbon chain is long and the other is short; this alignment may be limited to the shorter chain having but 1 or 2 carbons. As the length of the shorter chain increases, its hydrophobic nature increases and such a molecule will orient itself approximately as shown in structure *B*. Here the tendency of the shorter hydrocarbon chains to align themselves parallel to the interface results in these short chains partially overlying the adjacent ester group. Further lengthening of the hydrocarbon chain, beyond 4 or 5 carbon atoms, results in the "hairpin" orientation of structure *C*.

The members of Rideal's laboratory have studied the alkaline hydrolysis of a monomolecular film of monobasic esters (14) or of glycerides as well as monobasic esters (15). The relative rates of hydrolysis were consistent with the surface orientation patterns proposed by Adam. A further refinement of the structure at the interface was proposed from a consideration of the apparent dipole moments of the ester group.

Although no direct evidence is available, it is reasonable to assume the same configurations at an oil/water interface as have been proposed for the expanded film at an air/water interface. The relative rates of hydrolysis as a function of the alkyl group that we have observed (Table 4) are consistent with such a pattern of orientation. Methyl esters have the *A* configuration and hence would be hydrolyzed fairly rapidly (Fig. 2; *A*-1). The slow splitting of the ethyl esters is due to the extension of the ethyl group into the aqueous phase to the extent that it hinders the approach of the enzyme to the ester linkage (Fig. 2; *A*-2). This would be similar to the fourfold decrease in chemical hydrolysis between methyl and ethyl stearate that has been reported (14, 15). Hydrolysis of esters of the *B* configuration is slowed by

the overlaying of the ester groups by the alkyl chain of the adjacent ester, which once again interferes (but in a different way) with the contact between the enzyme in the aqueous phase and the ester linkage. As the chain length increases from 3 to 4 to 5 carbon atoms, this hindrance would become more effective. Since the mechanism of inhibition is not the same as in the *A*-2 structure, the rates of hydrolysis would not necessarily form a continuous series as the chain length is increased from 1 to 5 carbon atoms. When the alkyl chain contains more than 5 carbon atoms, the compound assumes the *C* configuration and is hydrolyzed readily because the ester group is advantageously placed for the action of the enzyme. The slower rate when the alcohol has 12 or more carbons poses a special problem that is being investigated.

The hydrolysis pattern imparted by the fatty acid moiety (Table 4) is not consistent with the interfacial configuration described above. Moreover, Alexander and Rideal (15) found the only acetate ester they studied to be split extremely rapidly by alkali at an air/water interface, whereas we found all of the acetate esters to be hydrolyzed very slowly by the enzyme. Recently we have been studying esters of octadecenoic acids in which the double bond is located in various positions. If one considers the effective chain length of these unsaturated fatty acids to be from the carboxyl group to the double bond, the relative rates of hydrolysis are quite similar to those seen in Table 4. Because of these considerations we are inclined to believe that the pattern of hydrolysis imparted by the fatty acid component is a reflection of enzyme–substrate specificity. However, the possibility that the primary effect of acyl chain length is due to substrate orientation cannot be ruled out.

Schulman (16) determined the rate of hydrolysis by lipase of nine of the esters that we have studied. He interpreted the relative rates solely in terms of the orientation of the substrate as discussed above. His values and ours are in only fair agreement. This may be due to differences in experimental technique in that he made but a single measurement at the end of 24 hr and his incubation mixtures were apparently not stirred.

When other workers have used simple triglycerides as the substrate, the maximum rate of hydrolysis was obtained when the fatty acids had a short chain. Thus Weinstein and Wynne (3) and Wills (9) found tripropionin to be split most rapidly, whereas Sobotka and Glick (4), Schonheyder and Volqvartz (7) and Entressangles, Pasero, Savary, Sarda, and Desnuelle (8) obtained the maximum rate of splitting with tributyrin as the substrate. Although we too find butyrate esters to be split rapidly, this rate is usually exceeded by that of the corresponding dodecanoate ester. The considerably slower rates observed by other workers for the triglycerides of these longer-chain fatty acid esters may be due to their being solids rather than liquids. The important effect of temperature of the incubation mixture as related to the melting point of the substrate has been reported by Balls et al. (5). They pointed out that if behavior only at low temperatures were considered, pancreatic lipase would be considered to be specific for glycerides of short-chain fatty acids, whereas at higher temperatures the maximum rate of splitting is attained with glycerides of acids containing from 7 to 10 carbon atoms. Unfortunately their incubations were carried out without agitation, so diffusion could have been the rate-limiting step. Savary and Desnuelle (17) found oleic and lauric acids to be split at the same rate from randomized triglycerides containing these two acids. They point out some of the complications that the melting point of the ester can impart to studies of substrate specificity. The two factors of diffusion and melting point could explain the divergent results for the rate of splitting of various substrates noted in the review by Wills (18).

The use of markedly different substrates, such as aromatic alcohols, makes comparisons even more difficult. However, it should be noted that Kramer et al. (19), in their studies with naphthyl esters, found the maximum rate of splitting by lipase when the chain length of the fatty acid was 9 carbons.

Interpretation of the results obtained in our study would have been greatly facilitated if adequate methods for determining the orientation of these esters at an oil/water interface had been available. Unfortunately there is no accepted method. As a consequence we cannot be certain that the explanations we have proposed for the effect of the alcohol and acid moieties are correct. They are, however, consistent with the limited information that is available, although most of these data have been obtained for the air/water interface.

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References

1. Mattson, F. H., and L. W. Beck. 1956. *J. Biol. Chem.* **219:** 735.
2. Sarda, L., and P. Desnuelle. 1958. *Biochim. Biophys. Acta.* **30:** 513.
3. Weinstein, S. S., and A. M. Wynne. 1936. *J. Biol. Chem.* **112:** 641.
4. Sobotka, H., and D. Glick. 1934. *J. Biol. Chem.* **105:** 199.
5. Balls, A. K., M. B. Matlack, and I. W. Tucker. 1938. *J. Biol. Chem.* **122:** 125.
6. Balls, A. K., and M. B. Matlack. 1938. *J. Biol. Chem.* **123:** 679.
7. Schonheyder, F., and K. Volqvartz. 1944. *Enzymologia.* **11:** 178.
8. Entressangles, B., L. Pasero, P. Savary, L. Sarda, and P. Desnuelle. 1961. *Bull. Soc. Chim. Biol.* **43:** 581.

9. Wills, E. D. 1961. *In* The Enzymes of Lipid Metabolism. P. Desnuelle, editor. Pergamon Press, New York. 13.
10. Mattson, F. H., and R. A. Volpenhein. 1966. *J. Lipid Res.* **7:** 536.
11. Mattson, F. H., and R. A. Volpenhein. 1966. *J. Amer. Oil Chem. Soc.* **43:** 286.
12. Hückel, W. 1958. *In* Theoretical Principles of Organic Chemistry. F. H. Rathmann, translator. Elsevier, New York. **2:** 736.
13. Adam, N. K. 1929. *Proc. Roy. Soc. Ser. A.* **126:** 366.
14. Alexander, A. E., and J. H. Schulman. 1937. *Proc. Roy. Soc. A.* **161:** 115.
15. Alexander, A. E., and E. K. Rideal. 1937. *Proc. Roy. Soc. A.* **163:** 70.
16. Schulman, J. H. 1941. *Trans. Faraday Soc.* **37:** 134.
17. Savary, P., and P. Desnuelle. 1956. *Biochim. Biophys. Acta.* **21:** 349.
18. Wills, E. D. 1965. *In* Advances in Lipid Research. R. Paoletti and D. Kritchevsky, editors. Academic Press, New York. **3:** 209.
19. Kramer, S. P., L. D. Aronson, M. G. Rosenfeld, M. D. Sulkin, A. Chang, and A. M. Seligman. 1963. *Arch. Biochem. Biophys.* **102:** 1.