BBA 65744

SUBSTRATE SPECIFICITY OF PANCREATIC LIPASE

H. BROCKERHOFF

Fisheries Research Board of Canada, Halifax Laboratory, Halifax, Nova Scotia (Canada)

(Received December 6th, 1967)
(Revised manuscript received February 16th, 1968)

SUMMARY

1. The hydrolysis of esters of oleic acid by pancreatic lipase (glycerol-ester hydrolase, EC 3.1.1.3) depends on the nature of the alcohol. Two factors appear to influence the speed of the reaction: an inductive effect, and steric hindrance.

2. Lipolysis is promoted by electrophilic substituents, as might be expected for a reaction involving nucleophilic attack on the carbonyl; *e.g.* oleyl oleate is split slowly, 2-fluoroethyl oleate or *p*-nitrobenzyl oleate rapidly. This inductive effect explains the following sequence: speed of lipolysis: triglyceride > 1,2-diglyceride > 1,3-diglyceride > 1-monoglyceride > 2-monoglyceride. It probably also regulates the attack of pancreatic lipase on different phospholipids.

3. Bulkiness of the carbinol group inhibits lipolysis: vinyl oleate is hydrolysed, but isopropyl or phenyl oleate are not. This steric effect explains the specificity of the lipase for the α-chains of triglycerides. Electron-withdrawing substituents can counteract this hindrance; *e.g.* 1,3-difluoroisopropyl oleate and *p*-nitrophenyl oleate are slowly hydrolysed.

4. The degree of hydration of the alcohol may be a third factor in lipolysis; *e.g.* trifluoroethyl ester reacts more slowly than monofluoroethyl ester, although the groups occupy similar space. This points to a change in the structure of the interphase.

5. It is suggested that lipases differ from other esterases in being comparatively weak nucleophilic agents.

INTRODUCTION

The natural substrate for pancreatic lipase (glycerol-ester hydrolase, EC 3.1.1.3) is triglyceride. The ester bonds in the α-positions are hydrolysed; α,β-diglycerides are formed; these are broken down, at a slower rate, to β-monoglycerides; and glycerol is released very slowly from the (isomerized) α-monoglycerides[1].

Triglyceride is the most active substrate known, but other esters of fatty acids may also be attacked by pancreatic lipase. The rate for methyl oleate has been reported as 1/30 of that of triglyceride[2].

In this paper, an attempt is made to explain the course of lipolysis and the

specificity of the lipase by comparing the structures and activities of different substrates. A part of this work was reported at a meeting[15].

MATERIALS AND METHODS

Substrates

The alcohols were bought from Aldrich Chemical Company, Milwaukee, Wisc., or from K and K Laboratories, Plainview, N.Y. They were assayed for purity by gas–liquid chromatography on a column with a polar stationary phase (ethylene glycol succinate–silicone copolymere) and on a porous column (Porapak, Waters Associates Inc., 61 Fountain St., Framingham, Mass., U.S.A.). If necessary they were purified by fractional distillation and by chromatography on silicic acid, and only preparations of higher than 97% purity were used. The important secondary alcohol, 1,3-difluoroisopropanol, was prepared better than 99% pure.

The alcohols were esterified with a slight excess of oleoyl chloride (from 99% *plus* oleic acid, from the Hormel Institute, Austin, Minn., and oxalyl chloride) in ether–pyridine. The esters were purifed by passage through aluminum oxide in ether. Their purity was checked by thin-layer chromatography.

Vinyl oleate was bought from K and K Laboratories and purified by passage through silicic acid and aluminum oxide. The product gave only one spot on thin-layer silicic acid. Gas–liquid chromatography showed 83% vinyl oleate; the rest was vinyl palmitate and other analogues. Diphenyl phosphatidate was prepared from 1,2-diglyceride, prepared from egg lecithin with phospholipase C (EC 3.1.4.3)[3], and diphenyl chlorophosphate in ether–pyridine. 3,4-Dichlorobenzyl alcohol was prepared by hydrogenation (H_2, PtO_2) of the corresponding aldehyde (Aldrich Chem. Co.). The product was homogeneous as shown by thin-layer chromatography. Acylation with oleoyl chloride yielded a homogeneous ester.

Commercial triolein was purified by chromatography on silicic acid and aluminum oxide.

Pancreatic lipase

Commercial pancreatic powder (Steapsin, Sigma Chemical Co., St. Louis, Mo.) was extracted and separated on Sephadex G-200 (refs. 4, 5). The first peak that appears contains the "fast" lipase of SARDA *et al.*[5], a multimolecular aggregate of lipase and phospholipid. The preparations showed activities of 200–250 (μmoles fatty acid released per min per mg protein), as determined according to the method of DESNUELLE, CONSTANTIN AND BALDY[6], and contained, therefore, only a few percent active lipase—pure pancreatic lipase has an activity of about 7000 (ref. 5). However, since all the substrates were insoluble, there could be no interference from other esterases in the assays. The only other enzymes that could have hydrolysed a lipid are phospholipase (EC 3.1.1.4) and cholesterol esterase (EC 3.1.1.13). As for the phospholipase, its relative activity under our conditions was less than 0.01 (triglyceride = 1.00), and this activity may have been due to lipase rather than phospholipase[7]. As for cholesterol esterase, this enzyme is inhibited by diethyl-*p*-nitrophenyl phosphate in solution, and it is dependent on bile salts[8,9]. In contrast, the activity of our preparation against secondary and phenolic esters was not lowered by the organic phosphate nor increased by bile salt.

Several determinations, especially that of the most highly active secondary ester,

were also done with a pure preparation of pancreatic lipase[5], a gift from Dr. G. BENZONANA.

Assays

Assays for the more active substrates were done by continuous titration of the fatty acid with an automatic recording titrator. The ester, 0.2 ml, was homogenized, with the help of a sonifier, for 3 min at 20 kcycles/sec, in 3 ml of 0.8 M NaCl and 0.1 M $CaCl_2$ in water, with 1% gum arabic as an emulsifier. Stirring was done by bubbling nitrogen through the homogenate at a rate sufficient to obtain maximal velocity of hydrolysis. The pH was 8.0, the temperature 18–20°. All assays were preceded and followed by assays of triolein. The linear slopes during the first minutes of the reactions were measured.

When the activity of the substrate was less than 1/10 that of triolein, the assays were run by shaking the ester, 0.1–0.3 ml, with lipase in 10 ml buffer (pH 8.0) (0.8 M NaCl, 0.1 M $CaCl_2$, 0.1 M Tris). The reactions were stopped, after 30 or 60 min, with 1 ml of 0.5 M H_2SO_4. Chloroform, 8 ml, was then added, the shaking was continued for 10 min, the chloroform was then collected with a syringe, and the fatty acids were titrated with 0.025 M NaOH, with bromothymol blue as an indicator. Triolein, as a standard, was assayed under the same conditions.

Accuracy

The reproducibility for both methods of analysis was within 10%. When the same ester (*O*-hexyl ethylene glycol, ethylene glycol, benzyl ester) was assayed in both systems, the agreement was also within 10%. In the tables, all activities refer to the speed of hydrolysis of triolein, set at 1.00.

Optimal substrate concentration must be used in a comparative study so that maximal velocities can be compared. (In lipolysis, "concentration" refers to the total surface of the substrate per volume, not to its weight[10].) In the assays with continuous titration the volumes were varied between 3 and 15 ml, and it was established for several substrates (triglyceride, benzyl, *p*-chlorobenzyl, 2-fluoroethyl ester) that the concentration finally used, 0.2 ml homogenized in 3 ml, was at least 5 times higher than that required for optimal velocity.

In the assays for the substrates of low activity, independence of the substrate concentration was established by varying the amount of substrate (0.1–0.3 ml) and the degree of agitation. Faster shaking increased the rate of hydrolysis, probably by providing better emulsification, and for the assays the mixtures were shaken at a rate 2 times higher than that required for optimal hydrolysis[9]. Several substrates (methyl, 1,3-difluoroisopropyl, benzyl ester) were also homogenized with the help of a sonifier. This procedure, which leads to a manifold increase of substrate surface, did not increase the activities.

With the substrates that were digested for 30 or 60 min there arose the problem of proportional hydrolysis during the 30–60 min of the reaction. Two substrates, *p*-chlorobenzyl and 2-fluoroethyl oleate, were compared with triolein at 5, 10, 20, 40, and 60 min. The velocities diminished at equal rates. Two factors are probably responsible for the decrease in activity: a deterioration of the enzyme during the time of reaction, and an inhibition by fatty acid. (The alcohols of the most interesting hindered substrates, 1,3-difluoroisopropanol and *p*-nitrophenol, did not inhibit at the expected

concentrations.) The inhibition by fatty acids is probably small in the presence of sufficient calcium, but it would affect the highly active substrates more than the slow ones. Consequently, the lower values in the tables may be too high in comparison with the standard, triolein, when assayed under the same conditions. In fact, the determination of the initial speed of lipolysis of the secondary ester, 1,3-difluoroisopropyl oleate, in an emulsion with desoxycholate and measured by continuous titration[10], showed an activity 50% lower than that given in Table III; but the different conditions may also be blamed for this difference. Everything considered, it should be stated that the data in the tables represent the correct relative, though not absolute values; *e.g.* it can be stated with confidence that the rate of lipolysis of isopropyl oleate < 1,3-difluoroisopropyl < *O*-hexyl ethylene glycol < benzyl oleate.

RESULTS AND DISCUSSION

The inductive effect

Lipolysis starts, we can assume, with a nucleophilic group of the enzyme attacking the carbonyl carbon of the substrate, and if this is the rate-limiting step, we can expect that the reaction will become faster if the carbon is made more electrophilic. This is a principle that governs the alkaline hydrolysis of esters in a single phase, and if it also holds for enzymatic lipolysis we should be able to demonstrate this with the help of differently substituted substrates. Table I, which is taken from a textbook of physical organic chemistry[11] shows some possible substituents.

TABLE I

HAMMET CONSTANTS σ FOR meta-BOUND SUBSTITUENTS COMPILED FROM HINE[11]

Substituent	σ	*Substituent*	σ
O^-	−0.71	Cl	+0.37
CH_3	−0.07	Br	+0.39
OH	−0.002	$CO_2C_2H_5$	+0.40
OCH_3	+0.12	CF_3	+0.43
C_6H_5	+0.22	CN	+0.68
F	+0.34	NO_2	+0.71

The σ is the substituent constant of the Hammet equation; it is a measure of the ability of the substituent to change the electron density at a neighbouring reaction centre[11]. σ can be determined by measuring the dissociation constants of substituted benzoic acids, and if the substituents are meta-bound, as the ones in Table I, then σ is a measure of the inductive effect of the substituent. A negative value means that the group is electron-donating, relative to hydrogen; the positive ones are electron-withdrawing. We can then expect the phenyl group, the halogens, ester groups, cyano and nitro groups to accelerate lipolysis, whereas hydroxyl should inhibit, more or less, depending on its ionization.

Table II shows the speed of hydrolysis of oleic esters by pancreatic lipase. The aliphatic groups, and the hydroxyl, keep the activity low. Alkoxy, halogens and the cyanide group increase it, so does the phenyl group in benzyl alcohol, and when we

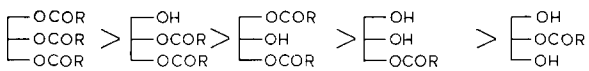
TABLE II

SPEED OF HYDROLYSIS OF ESTERS OF OLEIC ACID, RELATIVE TO TRIOLEIN ≡ 1.00

Triolein	1.00	FCH_2CH_2O-	1.2
$HOCH_2CH_2O$-	0.05	$ClCH_2CH_2O$-	0.25
Oleyl–	0.08	$BrCH_2CH_2O$-	0.16
CH_3O–	0.07	$NCCH_2O$–	0.80
$C_6H_{13}OCH_2CH_2O$-	0.16		
		$C_6H_5CH_2O$–	0.27
CH_2O-			
|	0.27	*p*-$ClC_6H_4CH_2O$–	1.3
CH_2O–			
		p-$NO_2C_6H_4CH_2O$-	1.4

introduce Cl or NO_2 we get a high activity. The ethylene glycol dioleate fits into this picture; it shows the activation by a neighbouring electrophilic ester group.

Table II suggests that the speed of lipolysis depends on the inductive effect of substituents in the alcohol. With this effect we can explain why diglycerides and monoglycerides accumulate during pancreatic lipolysis. The differences in the speed of lipolysis:



can be interpreted as the result of activation or deactivation by neighbouring ester or hydroxyl groups. (In addition there is steric hindrance in the last substrate.)

A substituent in the alcohol moiety which makes the carbonyl group more electrophilic will also make the anion of the alcohol more stable, and our results might also be discussed in terms of the stability of "leaving groups". However, this effect is in general unimportant compared to the activation of the carbonyl[11].

Hydrolysis of phospholipids

It has been shown by DE HAAS, SARDA AND ROGER[7] that the α-bound fatty acid in lecithin is attacked by lipase, though very slowly. It is probable that the structure of the lecithin micelle is in part responsible for this slowness of hydrolysis; but if we consider only the inductive effect we can see that the phosphoryl choline deactivates the ester group because the phosphate has a high electron density and a negative charge. From deacylation experiments it is known that the phosphoryl choline is much less electrophilic than the carboxyl ester group: it is possible to attack the carbonyls with a nucleophilic reagent, *e.g.* hydroxy or methoxy ion, without disturbing the phosphate group[12]. Removal of the negative charge on the phosphate should diminish the inhibition, and this was demonstrated with diphenyl phosphatidate, which had an activity of 0.08 in our assay, as compared to lecithin with an activity of less than 0.01; it can be predicted that a β-lecithin, with the deactivating group closer to the ester bond, will react even slower than lecithin. This is, indeed, the case (G. H. DE HAAS, personal communication).

Steric hindrance

The inductive effect does not explain why lipolysis does not start at the β-ester

of triglyceride whose carbonyl must be even more electrophilic than those in α since it is flanked by two activating groups. The reason seems to be steric hindrance. Since the activities in the experiments of Table III were so low, the actual release of fatty acids was always checked by thin-layer chromatography. We see that isopropyl oleate is almost completely resistant to pancreatic lipase, and so is phenyl oleate. In the isopropyl ester it must be steric hindrance that inhibits the reaction, rather than the

TABLE III

LIPOLYSIS OF SECONDARY AND PHENOLIC OLEIC ESTERS

Triolein ≡	*1.00*	CH_3O–	*0.07*
$(CH_3)_2CHO$–	0.00	C_6H_5O–	0.00
	(<0.003)	β-naphthyl–	0.02
$(ClCH_2)_2CHO$–	0.01	p-ClC_6H_4O–	0.01
$(FCH_2)_2CHO$–	0.05	p-$NO_2C_6H_4O$–	0.07
$(F_3C)_2CHO$–	0.01	CH_2=CHO–	0.45

influence of the two deactivating methyl groups, because substitution with fluorine, which is highly activating (Table II), gives a substrate with still very low activity (Table III). However, 1,3-difluoroisopropyl oleate, and *p*-nitrophenyl oleate, are definitely split by the lipase. This was confirmed for the secondary ester in an experiment with the highly purified lipase of SARDA *et al.*[5].

The effect of steric hindrance appears in the alkaline hydrolysis of esters in solution. In alkaline hydrolysis, however (Table IV taken from ref. 11), the deactivation from primary to secondary alcohol is not as great as in lipolysis. The reason is, perhaps, that in solution the nucleophilic agent can approach the carbonyl from many directions but at the interphase the freedom of the ester group is restricted and the active configuration of the enzyme–ester complex is rigid. The change from primary to secondary ester at the interphase is perhaps comparable to the step from secondary to tertiary ester in solution. This effect is much greater (Table IV).

TABLE IV

RELATIVE RATES OF ALKALINE ESTER HYDROLYSIS IN WATER AT 25° COMPILED FROM HINE

$CH_3CO_2CH_3$	1.00	$CH_3CO_2CH(CH_3)_2$	0.15
$CH_3CO_2C_2H_5$	0.60	$CH_3CO_2C(CH_3)_3$	0.008

We can now answer the question of whether lipase can hydrolyse β-monoglycerides. Isopropyl oleate is hydrolysed at least 300 times slower than triglyceride (Table III). Substitution with hydroxyl in positions 1 and 3 would inhibit the reactivity even further. An α-monoglyceride, however, should react only slightly slower than a glycol monoester (Table II), *i.e.* 20 times slower than triglyceride, or at least 15 times faster than the β-isomer. This means, if hydrolysis of β-monoglyceride by pancreatic lipase occurs at all, it must be quite negligible; results obtained by ENTRESSANGLES, SARI AND DESNUELLE[13] agree with this conclusion.

Changes in the structure of the interphase

The substituents we introduced will not only exert their inductive or steric influence on the ester group, they will also impose their characteristics on the structure of the interphase. For instance, they will react physicochemically in different ways with water or with the lipid phase. The previous results have demonstrated the action of the inductive effect, but Table V shows that this effect is not additive, as it would be for alkaline hydrolysis in a single phase. We expect increasing activity with increasing substitution, but this is not found. In some cases there may be steric hindrance

TABLE V

SPEED OF LIPOLYSIS OF ESTERS OF OLEIC ACID, RELATIVE TO TRIOLEIN ≡ 1.00

FCH_2CH_2O-	1.2	$C_6H_5CH_2O-$	0.27
F_2CHCH_2O-	0.82	4-$ClC_6H_4CH_2O-$	1.3
F_3CCH_2O-	0.32	3,4-$Cl_2C_6H_3CH_2O-$	1.1
$ClCH_2CH_2O-$	0.25	$F_5C_6CH_2O-$	0.04
Cl_2CHCH_2O-	0.45	2,6-$Cl_2C_6H_3CH_2O-$	0.00
Cl_3CCH_2O-	0.05		

to blame, for instance in the trichloroethyl oleate, and especially in the 2,6-dichlorobenzyl oleate. Fluorine atoms, however, are only slightly larger than hydrogen. It is therefore surprising that trifluoroethyl oleate is less active than the monofluoroethyl ester. Perhaps the shell of water is more tightly bound around the trifluoro group and this makes it more difficult for the enzyme to penetrate to the carbinol group.

Characteristics of pancreatic lipase

The experimental results support the following speculations. The accumulation of diglycerides and monoglycerides during the course of lipolysis and the "positional" specificity of pancreatic lipase can be explained with the electrophilic and steric properties of the substrates. It is not necessary to postulate anything beyond a one-point nucleophilic attack of the enzyme on the substrate. This is not to say that a two-point, or multiple, attachment does not actually take place, and we do not touch the question of the adsorption of the enzyme to the substrate or of the actual mechanism of the nucleophilic reaction. It is implied, however, that a simple nucleophilic agent, *e.g.* an alkoxy ion, if it could be made adsorbant to the interphase and self-regenerating, would show the same substrate specificity and positional action against lipids as does pancreatic lipase.

Pancreatic lipase acts only on a lipid–water interphase. This property has been used to define lipases[1] and distinguish them from other esterases, and it has been suggested that lipases may unfold to expose their active centres only in contact with the lipid. An alternative classification would describe the lipases as weaker nucleophilic enzymes than other esterases. An esterase can hydrolyse a simple carboxylic ester in aqueous solution, where the carbonyl group is surrounded by water molecules. A lipase, however, needs for proper functioning a carbonyl group activated by neighbouring ester groups, and it is too weak to penetrate the shell of a highly polar solvent. At the interphase, however, the substrate is sufficiently desolvated to allow the approach of

a weaker nucleophilic agent. An "inhibition of lipolysis by water" has been suggested by SCOTT[14].

ACKNOWLEDGEMENTS

Several experiments were carried out at the Institut de Chimie Biologique at Marseille. I wish to thank the Director, Dr. P. DESNUELLE, for his hospitality, and Drs. G. BENZONANA and M. LAZDUNSKI for help and advice.

REFERENCES

1 P. DESNUELLE, *Advan. Enzymol.*, 23 (1961) 129.
2 L. SARDA AND P. DESNUELLE, *Biochim. Biophys. Acta*, 30 (1958) 513.
3 D. J. HANAHAN AND R. VERCAMER, *J. Am. Chem. Soc.*, 76 (1954) 1804.
4 B. GELOTTE, *Acta Chem. Scand.*, 18 (1964) 1283.
5 L. SARDA, M. F. MAYLIÉ, J. ROGER AND P. DESNUELLE, *Biochim. Biophys. Acta*, 89 1964) 183.
6 P. DESNUELLE, M. J. CONSTANTIN AND J. BALDY, *Bull. Soc. Chim. Biol.*, 37 (1955) 285.
7 G. H. DE HAAS, L. SARDA AND J. ROGER, *Biochim. Biophys. Acta*, 106 (1965) 638.
8 L. SWELL, R. F. DAILY, H. FIELD, JR. AND C. R. TREADWELL, *Arch. Biochem., Biophys.*, 59 (1955) 393.
9 F. H. MATTSON AND R. A. VOLPENHEIN, *J. Lipid. Res.*, 7 (1966) 536.
10 G. BENZONANA AND P. DESNUELLE, *Biochim. Biophys. Acta*, 125 (1966) 55.
11 J. HINE, *Physical Organic Chemistry*, McGraw-Hill, New York, 1956.
12 R. M. C. DAWSON, *Biochim. Biophys. Acta*, 14 (1954) 374.
13 B. ENTRESSANGLES, H. SARI AND P. DESNUELLE, *Biochim. Biophys. Acta*, 125 (1966) 597.
14 J. E. SCOTT, *Nature*, 177 (1953) 777.
15 H. BROCKERHOFF, *Federation Proc.*, Abstr., 26 (1967) 606.