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# SUBSTRATE SPECIFICITY OF PANCREATIC LIPASE

## INFLUENCE OF THE STRUCTURE OF FATTY ACIDS ON THE REACTIVITY OF ESTERS

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SUMMARY

Various esters of monoenoic, polyenoic, methyl-branched, and $\omega$-cyclohexyl or $\omega$-phenyl substituted acids and also esters of aliphatic acids of various chain lengths were hydrolysed by porcine pancreatic lipase, and their maximal rates of lipolysis were compared to the rates of the corresponding oleates.

1. Substituents or unsaturation on Carbon 2–5 led to a relative resistance of the esters against lipase. *Cis* and *trans* unsaturation and the one triple bond tested inhibited lipolysis by comparable degrees. Introduction of additional but more remote double bonds, as in marine polyenoic acids, introduced no additional resistance. Between unsaturation at $\Delta^{2,3}$ and $\Delta^{5,6}$, the resistance does not depend in a coherent manner on the actual position of the double bond. $\Delta^{2,3}$-acids or $\Delta^{4,5}$-acids may in fact be slightly better substrates than $\Delta^{5,6}$-acids.

2. Compared to a monomethyl-branched acid, multiple branching introduced further resistance. Cyclohexylacetic acid was almost completely resistant. Inhibition by $\omega$-phenyl groups disappeared gradually as the chain was lengthened to carbon 6. The $\omega$-phenyl octanoates had an abnormally high rate of lipolysis, two to three times that of oleate.

3. Lipolysis rates of esters of straight-chain saturated acids increased from $C_2$ to a maximum at $C_4$, decreased suddenly at $C_5$ and then increased until around $C_9$ the rates of the oleates were approached.

4. Formates were attacked by lipase at rates comparable to those of oleates.

5. It is concluded that the resistance of unsaturated or branched acids is due to steric hindrance during the formation of the activated complex. This hindrance disappears largely for structures after $C_5$. The saturated $C_4$-chain seems to be optimally adaptable to the enzyme. The adsorption of the enzyme to the interphase should be viewed as a process preceding and separate from the formation of the substrate–enzyme complex. In this complex the esters are fixed to the enzyme in a two-dimensional orientation, probably along the axis carbonyl carbon–ether oxygen of the carboxyl ester group. Three-point fixation is a possibility. It is suggested that fixation and activation of the substrate is achieved by hydrogen bonding to both carbonyl

and ether oxygen, and that the leaving alkoxy group is received by a labile hydrogen of the enzyme.

## INTRODUCTION

The speed of hydrolysis of an ester by pancreatic lipase (EC 3.1.1.3) depends on the structure of the ester. Several structural factors can be isolated. Electrophilicity of the alcohol moiety of a substrate promotes hydrolysis; steric hindrance (secondary, phenolic alcohols) retards it[1]. Hydrophilicity of the whole molecule inhibits lipolysis, even when the substrate is offered as an emulsion[2]. As for the acidic moiety of the substrate, it has often been reported that short-chain acids, and especially propionic and butyric, accelerate lipolysis (reviewed by MATTSON AND VOLPENHEIM[3]), but it has also been suggested that the overall structure of a glyceride rather than the chain length of a particular acid determines the relative rates of hydrolysis[4]. However, MATTSON AND VOLPENHEIM[3] have recently conducted experiments with monoesters which confirm that chain lengths do influence reaction rates.

Steric hindrance seems to prevent the lipolysis of 2,2-dimethylstearic acid esters[5]. The polyenoic acids of marine oils which have double bonds in position 4 or 5 from the carboxyl group are relatively resistant toward lipase[6–8]. Similar resistance is shown by *trans*-3-enoic acids[9]. The marine acids have been the subject of a detailed study by BOTTINO *et al.*[8].

The present study deals with monoenoic and polyenoic acids, methyl-branched acids, and $\omega$-cyclohexyl and $\omega$-phenyl acids with the functional groups at varying distances from the carboxyl group. Care was taken to keep the alcohol moieties constant. This cannot be done in working with triglycerides; in triolein, for instance, the alcohol is glycerol dioleate, in triacetin, glycerol diacetate, two alcohols of vastly different physicochemical properties. Appropriate esters of other alcohols were therefore prepared. For most substrates *p*-chlorobenzyl alcohol or 2-fluoroethanol were employed, alcohols which are sufficiently electrophilic to yield oleates of an activity comparable to that of triolein[1]. All esters used were liquid at the temperature of assay.

## MATERIALS AND METHODS

*p*-Chlorobenzyl alcohol (Aldrich Chemical Co., Cedar Knolls, N.J.) was recrystallized from ethanol–water (m.p. 73°). 2-Fluoroethanol (K and K Laboratories, Plainview, N.Y.) was redistilled on a rotating band column (b.p. 103°). Cinnamyl alcohol was bought from Fisher Scientific Co., Montreal, 2-hexoxyethanol (ethylene glycol hexyl ether) from K and K Laboratories.

The sources of the acids are given in Table I. The acids were analysed by gas chromatography of their methyl, fluoroethyl or *p*-chlorophenyl esters. All substrates were better than 95% pure, with the exceptions listed in the table. The purity of the monoenoic acids of chain lengths 6–12 was verified by $AgNO_3$–$H_2SiO_3$ thin-layer chromatography. The configuration of the double bonds was established by infrared spectrometry.

Esters were usually prepared as follows. The acid was treated with a 2-fold

TABLE I

MAXIMAL VELOCITIES OF HYDROLYSIS OF ESTERS BY PANCREATIC LIPASE, EXPRESSED AS FRACTION OF THE VELOCITY OF THE CORRESPONDING ESTER OF OLEIC ACID UNDER THE SAME CONDITIONS, $V_{rel}$

The sources of acid are: (1) K and K Laboratories, Plainview, New York. (2) from the *p*-chlorobenzyl ester of 2-yne-8:1 by hydrogenation with LINDLAR[21] catalyst. (3) courtesy of Hopkins of the National Research Council, Ottawa[22]. From a mixture of 12:0 and 14:1, the monoene was isolated by column chromatography on $AgNO_3$–$H_2SiO_3$ (ref. 23). (4) The Hormel Institute, Austin, Minn. (5) Aldrich Chemical Co., Cedar Knolls, N.J. (6) Sorbic acid, practical. Fisher Scientific Co., Montreal, Quebec. (7) Both a technical grade (for the FEt ester) and a highly purified grade (99%, for both FEt and ClBzl esters) were tested (Hormel). The results for the different FEt esters were identical; (8) 99% arachidonic acid, Hormel. (9) 99% linoleic acid, Hormel. (10) 99% linoleic acid, Hormel. (11) Isobutyric acid, Fisher Scientific Co. (12) Isovaleric acid, Aldrich. (13) Phytanic acid, Analabs, Hamden, Conn. (14) Hydrogenation of the phenylpropiolic ester (Aldrich). (15) Oxidation of 6-phenyl hexanol-1 (K and K)[24]. (16) Formic acid, Fisher Scientific Co. (17) Acid chlorides, Fisher Scientific Co. (18) Ester purchased from K and K, purified on $Al_2O_3$.

| *Acid* | *Ester* | $V_{rel}$ | *Conditions* | *Source of acid* |
|---|---|---|---|---|
| *(A) Monounsaturated acids* | | | | |
| *t*2–6:1* | ClBzl | 0.04 | 25°, pH 8 | 1 |
| *t*2–8:1 | ClBzl | 0.10 | 25°, pH 8 | 1 |
| 2*y*–8:1* | ClBzl | 0.21; 0.22 | 25°, pH 8; 37°, pH 9 | 1 |
| *c*2–8:1 | ClBzl | 0.19 | 37°, pH 9 | 2 |
| *t*2–12:1 | FEt | 0.08 | 37°, pH 9 | 1 |
| *t*3–6:1 | ClBzl | 0.04 | 25°, pH 8 | 1 |
| *c*4–12:1 | FEt | 0.18 | 37°, pH 9 | 3 |
| *c*5–14:1 | ClBzl | 0.13 | 37°, pH 9 | 4 |
| *c*5–20:1 | FEt | 0.08 | 37°, pH 9 | 4 |
| *c*6–18:1 | FEt | 0.34 | 37°, pH 9 | 4 |
| *c*9–18:1 | Ester of ref. | 1.00 | —, — | 4 |
| 10–11:1 | ClBzl | 0.81 | 37°, pH 9 | 5 |
| *c*11–20:1 | FEt; ClBzl | 0.72; 0.82 | 37°, pH 9 | 4 |
| *c*11–22:1 | FEt | 0.61 | 37°, pH 9 | 4 |
| *(B) Polyunsaturated acids* | | | | |
| *t*2, *t*4–6:2* | ClBzl | 0.008 | 37°, pH 9 | 6 |
| *c*4, *c*7, *c*10, *c*13, *c*16, *c*19–22:6 | FEt; ClBzl | 0.20; 0.34 | 37°, pH 9 | 7 |
| *c*5, *c*8, *c*11, *c*14–20:4 | ClBzl | 0.09 | 37°, pH 9 | 8 |
| *c*5, *c*8, *c*11, *c*14 *c*17–20:5 | FEt; ClBzl | 0.13; 0.09 | 37°, pH 9 | 7 |
| *c*9, *c*12–18:2 | ClBzl | 0.85 | 37°, pH 9 | 9 |
| *c*9, *c*12, *c*15–18:3 | ClBzl | 0.89 | 37°, pH 9 | 10 |
| *(C) Methyl-branched acids* | | | | |
| 2-Me-$C_4$** | ClBzl | 0.01 | 29°, pH 8 | 11 |
| 3-Me-$C_5$ | ClBzl | 0.04 | 37°, pH 9 | 12 |
| 3 7,11,14-$Me_4$-$C_{20}$ | ClBzl | 0.006 | 37°, pH 9 | 13 |
| 4-Me-$C_6$ | ClBzl | 0.04 | 29°, pH 8 | 1 |
| 5-Me-$C_7$ | ClBzl | 0.37 | 29°, pH 8 | 1 |
| *(D) ω-Phenyl and ω-cyclohexyl acids* | | | | |
| Cyclohexyl-$C_2$ | FEt | 0.003 | 25°, pH 8 | 5 |
| Cyclohexyl-$C_3$ | FEt | 0.01 | 25°, pH 8 | 5 |
| Phenyl-$C_3$ | FEt | 0.04 | 25°, pH 8 | 14 |
| Phenyl-$C_4$ | ClBzl | 0.15 | 37°, pH 9 | 5 |
| Phenyl-$C_5$ | ClBzl | 0.20 | 37°, pH 9 | 5 |
| Phenyl-$C_6$ | FEt | 0.59 | 37°, pH 9 | 15 |
| Phenyl-$C_8$ | FEt; ClBzl | 3.4; 2.2 | 37°, pH 9 | 1 |
| Phenyl-$C_{10}$ | ClBzl | 0.98 | 37°, pH 9 | 1 |

TABLE I *(continued)*

| *Acid* | *Ester* | $V_{rel}$ | *Conditions* | *Source of acid* |
|---|---|---|---|---|
| *(E) Saturated straight-chain acids* | | | | |
| $C_1$ | ClBzl; Cinn | 0.59; 2.6 | 25°, pH 8 | 16 |
| $C_2$ | ClBzl | 0.36 | 25°, pH 8 | 17 |
| $C_3$ | ClBzl | 0.47 | 25°, pH 8 | 17 |
| $C_4$ | ClBzl | 1.13 | 37°, pH 9 | 17 |
| $C_5$ | ClBzl | 0.53 | 37°, pH 9 | 17 |
| $C_6$ | ClBzl | 0.65 | 37°, pH 9 | 17 |
| $C_7$ | ClBzl | 0.72 | 37°, pH 9 | 17 |
| $C_9$ | ClBzl | 0.92 | 37°, pH 9 | 1 |
| $C_2$ | HEt | 0.09 | 25°, pH 8 | 17 |
| $C_3$ | HEt | 0.28 | 25°, pH 8 | 17 |
| $C_4$ | HEt | 0.48 | 25°, pH 8 | 17 |
| $C_5$ | HEt | 0.44 | 25°, pH 8 | 17 |
| $C_7$ | HEt | 0.60 | 25°, pH 8 | 17 |
| $C_9$ | HEt | 0.94 | 25°, pH 8 | 1 |
| $C_2$ | Vinyl | 0.28 | 37°, pH 8 | 18 |
| $C_3$ | Vinyl | 0.85 | 37°, pH 8 | 18 |
| $C_4$ | Vinyl | 2.0 | 37°, pH 8 | 18 |
| $C_5$ | Vinyl | 0.65 | 37°, pH 8 | 18 |
| $C_6$ | Vinyl | 0.66 | 37°, pH 8 | 18 |
| $C_8$ | Vinyl | 1.07 | 37°, pH 8 | 18 |
| $C_{12}$ | Vinyl | 1.1 | 37°, pH 8 | 18 |

Abbreviations: ClBzl, *p*-chlorobenzyl ester; FEt, 2-fluoroethyl ester; Cinn, cinnamic ester; HEt, 2-hexoxyethyl ester.

* Nomenclature after HOLMAN[14]: configuration + position of double bond–chain length: number of double bonds; *y* = triple bond.

** Total carbon number.

excess of oxalyl chloride, with gentle warming, until the evolution of gas ceased (5–20 min). The excess of oxalyl chloride was removed in a rotary evaporator. 3–10 vol. of solvent (ether, alcohol-free chloroform; tetrahydrofurane for the preparation of fluoroethyl esters) and the calculated amount of alcohol were added, followed by 1.5 equivalents of pyridine. Solvents and pyridine were evaporated after 5 min, the residue was taken up in light petroleum (b.p. 30–60°)–diethyl ether (1:10 by vol.) (the pyridinium chloride need not be removed), placed on a column of neutral aluminum oxide[10], 4 g/g ester, and eluted with the same solvent system, which was then removed, together with any residual pyridine, in an evaporator. Commercially available acid chlorides were employed in the case of the lower saturated fatty acids ($C_2$–$C_7$). Formate esters were prepared by refluxing the alcohol with the 5-fold excess of formic acid for 4 h, evaporating the excess acid and water, dissolving the esters in ether, washing with water and drying with $Na_2SO_4$, followed by purification on aluminum oxide as above. All esters were checked for purity by thin-layer chromatography. They were usually prepared on the day before the enzymatic assay. If they were reused later, they were purified by passage through neutral aluminum oxide to remove any free acid. The vinyl esters were bought from K and K Laboratories and purified on aluminum oxide.

The enzyme, porcine pancreatic lipase, was prepared according to VERGER *et al.*[11]. It had a specific activity of 4500 ($\mu$moles · min$^{-1}$ · mg protein$^{-1}$) against triolein emulsified with gum arabic[12]. The purity of the preparations has been demonstrated by standard procedures and convincingly by its separation into two isoenzymes of equal activity[11].

The assay system was that described by BENZONANA AND DESNUELLE[13]; final concns.: 0.1 M NaCl, 0.5 mM $CaCl_2$, 4.5 mM sodium deoxycholate; total volume, 15 ml. The temperature was normally 37°, the pH 9.0, but many short chain esters were found to hydrolyse spontaneously under these conditions; temperature and pH were lowered in these cases as listed in Table I. The corresponding oleate was always measured under the same conditions. Substrates were added after sonic emulsification (20 kcycles, 50 W, 30 sec) with a 2-fold volume of 32 mM deoxycholate[13]. Assays were always sandwiched between control assays of the corresponding oleate. The fatty acids released by the action of the lipase were titrated continuously (Radiometer, Copenhagen). The reactions were carried out under nitrogen, with stirring at maximum speed. The recorded linear rates during the first few minutes were measured. Maximum velocities V were established by varying the amounts of substrate emulsion from 0.05 to 0.3 ml.

The specific activity of the pancreatic lipase in this assay was 3600 against triolein; 3600 against *p*-chlorobenzyl oleate; 1200 against 2-fluoroethyl oleate; 1100 against vinyl oleate. The reproducibility of assays for these esters was better than $\pm$ 10%. The accuracy of the relative rates of 0.1 or less (Table I) may be taken for $\pm$ 20%.

## RESULTS

### *Double bonds near the carboxyl group*

Double bonds in positions 2–5 (*i.e.* $\Delta^{2,3}$–$\Delta^{5,6}$) strongly inhibit lipolysis, but there seems to be little differentiation within this range; *c*2-8:1 (*i.e. cis*-octa-2-enoic acid; nomenclature according to HOLMAN[14], see footnote to Table I) is just as rapidly hydrolysed as *c*4-12:1 and even slightly faster than *c*5-14:1 and *c*5-20:1. The polyenoic acid with the first double bond in $\Delta^4$ is more easily removed than the $\Delta^5$-polyenoic acids; this is true for the fluoroethyl as well as for the chlorobenzyl ester. This result was confirmed for different preparations of the acid (see Table I); and it can also be recognized in the lipolysis of a triglyceride, seal oil[6]. Interestingly, this effect appears also in the monoenes: the *cis*-4-monoene is less resistant to lipase than the *cis*-5-monoenes. The chain length, however, may be an additional factor.

Only tentative conclusions can be drawn concerning *cis* or *trans* double bonds and chain length. *Cis* unsaturation may be less restricting than *trans* unsaturation, though the opposite might have been anticipated on theoretical grounds (see DISCUSSION, last paragraph). The triple bond in $\Delta^{2,3}$ behaves, in this regard, like a *cis* bond. Increasing the chain length of a monoenoic acid appears in general to increase the resistance to lipase slightly.

The highly unsaturated polyenoic acids are not more resistant to lipase than the monoenoic acids with the double bond in the position of the first double bond of polyenes. The exception is *t*2,*t*4-6:2, sorbic acid, in which the accumulation of two *trans* double bonds before $C_5$ makes for a very resistant substrate.

The hindrance disappears around carbon 6. Whereas the $\Delta^{5,6}$ acids show approx. 10-fold hindrance, the $\Delta^{6,7}$-acid (petroselinic acid) is only 3-fold hindered. The $\Delta^{7,8}$-polyenoic acid that occurs in seal oil shows no hindrance effect[6,8].

*Substitution near the carboxyl group*

Methyl groups in position 2, 3 and 4 inhibit strongly. Between substitution at 4 and 5 there is a 10-fold jump in activity. Introduction of further methyl groups after the carboxyl-proximal methyl, as seen in the example of phytanic acid, imposes further restrictions on lipolysis. This is in contrast to the lack of effect of the double bonds of polyenoic acids. In the cyclohexyl-phenyl series the inhibition disappears more gradually; at $C_6$ less than 2-fold restriction remains.

While $\omega$-phenyl-$C_6$ approaches the activity of oleate, and $\omega$-phenyl-$C_{10}$ equals it, we find a surprising anomaly for $\omega$-phenyl-$C_8$. The rates of lipolysis of both the fluoroethyl and the chlorobenzyl ester are much higher than that of the oleate or butyrate. As this result was so unexpected, special care was taken to assure the purity of the substrate. The methyl and fluoroethyl ester of the acid appeared homogeneous on gas chromatography. The substrates were pure on thin-layer chromatography. Two years after the first finding the two substrates were newly synthesized and assayed, with the same result. The high activity must be considered real, though unexplainable.

*Variations in chain length of acids*

In the *p*-chlorobenzyl ester and in the vinyl ester series we find that the rate of lipolysis increases from a low for $C_2$ to a maximum at $C_4$, decreases at $C_5$ and then climbs to nearly the level of the oleate at $C_9$.

The 2-hexoxyethyl ester series runs parallel to the other two, although the peak at $C_4$ is not very pronounced but rather like a shoulder. The short-chain esters of this series, derivatives of ethylene glycol, are probably quite hydrophilic; this may explain[2] the relatively low rates from $C_2$ to $C_4$.

The results agree very well with those of MATTSON AND VOLPENHEIN[3] in their work on the lipolysis of alkyl esters. These authors find a maximum at $C_4$ and the same peculiar minimum at $C_5$.

Esters of formic acid turned out to be very good substrates. The high relative value for the cinnamyl formate may result from the fact that the compound of reference, cinnamyl oleate, is a very poor substrate because the alcohol is so little activating. It is certain, however, that both formates are attacked by lipase with a speed approaching that for oleates and butyrates.

## DISCUSSION

Although it has not yet been demonstrated, we may assume that lipase, like other hydrolases, forms a substrate–enzyme complex, attacks its substrate as a nucleophile, is thereby acylated and transfers the acyl group to water. The structural features of the substrate intrude into this scheme and lead to different reaction rates. In a previous study on the influence of the alcoholic moiety[1], we concluded that the nucleophilic reaction is governed by the electrophilicity of the alcohol. The steric properties of the alcohol, which are very decisive as regards the reaction rate[1], must come to bear during the formation of the active complex.

*Structure of acids and reaction rate*

The only important rate-determining factor of the acids investigated here seems to be their steric structure. Inductive or mesomeric effects would fall off strongly, or abruptly, within a short distance from the carboxyl group. Instead, the restriction by double bonds, or substituents, disappears only after $C_5$ or $C_6$ and rather gradually than abruptly. The exact position or configuration of the restricting double bonds or branching does not seem to be critical. The principal requirement is perhaps that the chain can conveniently bend away from the enzyme. The freely flexible $C_4$-acid seems optimally adapted to the spatial environment, but the lower reaction rates of $C_2$ and $C_5$ are puzzling, and so is the peculiarly high rate of the $\omega$-phenyl octanoates. The explanation of these phenomena will probably have to await the elucidation of the structure of the enzyme.

*Course of lipolysis*

Since lipolysis takes place at the interphase of oil and water, it has been argued that different orientations of molecules in the interphase may account for different reaction rates. MATTSON AND VOLPENHEIN[3] discuss the possible orientations of the alcohol moieties of esters in this respect; but they believe that those patterns of hydrolysis imparted by the fatty acid components reflect enzyme–substrate specificity rather than orientation. Our results lead to the same conclusion. First, the reaction rates for substituted acids vary in a continuous manner; it is unlikely that the orientation of their esters in the interphase should be patterned in precisely the same gliding scheme. Second, the acids show analogous relative reaction rates, whether they are bound to diacylglycerol, fluoroethyl, hexoxyethyl, or *p*-chlorobenzyl alcohol. Again, it is unlikely that these different alcohols would allow the different acids to orient themselves in the same relative order.

Lipolysis at the interphase, then, must not be imagined as the enzyme battering against a wall of ester groups and splitting the ones most exposed. It is the function of the interphase, I suggest, to adsorb the enzyme and thus provide the highest possible concentration of both enzyme and substrate, and to provide a milieu of relative dehydration in which the ester groups are more easily approachable by the nucleophilic enzyme[2]. The enzymic reaction proper proceeds in the classical manner; the enzyme binds one molecule of substrate to form an "active complex". The affinity between substrate and enzyme is determined by the steric structures of enzyme and substrate.

We may summarize the hypothetical course of lipolysis and the factors that determine $V$:

$$E + S \rightleftharpoons [E + S]_{\text{adsorbed}} \rightleftharpoons ES \rightarrow EP_2 + P_1 \rightarrow E + P_2$$

The enzyme is physically adsorbed to the interphase. When the interfacial area is large enough to adsorb practically all enzyme molecules, we reach the limit of the measurable overall reaction rate, $V$, because the highest possible enzyme and substrate concentration is reached. In a modification of the concept of "unproductive binding"[15], we might speak of "preproductive binding". A fraction of all interfacial enzyme molecules is bound as substrate–enzyme complex; $V$ is proportional to the size of this fraction, and this depends on the structure of the substrate.

This scheme has implications for the interpretation of Michaelis constants. It

has been pointed out[16–18] that the $K_m$ of lipolysis should be measured in the dimension of surface per volume and not of concentration, and such a $K_m$ has been determined by BENZONANA AND DESNUELLE[18]. According to our scheme this $K_m$ is the dissociation constant of the enzyme–interphase complex; it has already been so understood by DIXON AND WEBB[19]. This implies that quite similar values of $K_m$ (defined as the interfacial area per volume binding half the enzyme) would result if the triglyceride would be replaced by a nonsubstrate like paraffin oil, or the lipase by a similar, but nonenzymatic protein. The $K_m$ measured as interphase per volume is, of course, quite correctly named, as it fulfils its function in the Michaelis–Menten equation[18]; but if it should turn out, as I think it may, that $K_m$ is dependent on the interfacial tension and potential, but independent of the structure of the substrate, then such a $K_m$ does not carry the traditional meaning as a measure of binding in a bimolecular enzyme–substrate complex.

No $K_m$ measurements were carried out in this study, but there were indications that the $K_m$ values for all insoluble substrates were indeed of comparable magnitude. The velocity $V$ of lipolysis increased always in similar steps between the addition of 0.05–0.1–0.2 ml of stock emulsion to the assay solution and reached a maximum around 0.3 ml. Assuming that all emulsions (which had been prepared under the same conditions) had the same interfacial area, we may conclude that the $K_m$ values must be very similar.

*Fixation of the substrate to the enzyme*

Our data support these suggestions: in the active complex, the substrate is bound to the enzyme in a two-dimensional orientation; fixation probably occurs at the ether–oxygen through hydrogen bonding; additional fixation at the carboxyl oxygen (three-point fixation) cannot be excluded; lipophilic bonding does not play an important role.

A two-dimensional fixation follows from steric considerations. Fig. 1 shows the structure of *p*-chlorobenzyl 3-cyclohexylpropionate. The nucleophilic attack occurs at the centre. We know that the left side of the formula (the *p*-chlorobenzyl group, also the very similar diglyceride group) imposes no steric hindrance, because the obviously unhindered highly electrophilic cyanomethyl of fluoroethyl esters show no higher activities; but the hindrance at the right side of the structure is severe. For example, *p*-chlorobenzyl butyrate is a very good substrate, but 2-fluoroethyl 3-cyclohexylpropionate is very resistant (Table I), although size and shape of the two com-

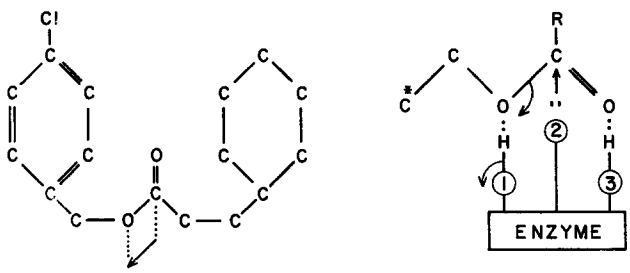


Fig. 1. Two-dimensional fixation of *p*-chlorobenzyl 3-cyclohexylpropionate to lipase.

Fig. 2. Hypothetical fixation and lipolysis of a glyceride.

pounds are nearly identical. That is to say, the substrate cannot rotate on the enzyme. Since the substituents on both sides can be changed in many ways and still yield active substrates, with the only common structural feature being the grouping C—O—C=O, it is probably the carbonyl carbon (the point of attack) and the ether oxygen that fix the orientation, as indicated in Fig. 1.

It can reasonably be assumed that the fixation of the ether oxygen is accomplished through hydrogen bonding (Fig. 2). Such bonding would also polarize the ester and facilitate nucleophilic attack at the carbonyl carbon (general acid catalysis). Furthermore, the labile hydrogen of the enzyme might well accept the alkoxy (or phenoxy) leaving group. This would obviate the immediate need for water to react with the highly nucleophilic leaving group and agree well with the hydrophobic character of lipolysis.

Fig. 2 shows the proposed fixation along 1–2 with a hydrogen bond at 1. It should be noted that a fixation along the carbonyl bond, 2–3, could not account for the steric hindrance effects, since the (permissible) rotation around this axis would allow accommodation of a bulky substituent in either the acidic or the alcoholic moiety. On the other hand, fixation at 3 in addition to fixation at 1, and also by hydrogen bonding, is quite possible. In fact, such a bond would help in further polarizing the carbonyl carbon atom. The fact that lipase does not differentiate between stereoisomeric triglycerides does not argue against the three-point fixation that would result from binding at 3. Stereospecificity of an enzymic reaction requires three-point fixation, but a given three-point fixation controls the configuration only at the center of the reaction, not at a position as remote as C* in a triglyceride. Fig. 2 shows this clearly.

The binding at 3 depicted in Fig. 2, although possible and even plausible, is still merely hypothetical and so far not bolstered by experimental evidence. There is, however, one more argument in its favor. Hydrogen bonding to ether oxygen, as it is postulated here at Point 1, is known to exist[20], but it is believed to be much weaker than hydrogen bonding to carbonyl oxygen. I therefore suggest that the first, and strongest, fixation of the substrate takes place at 3 (Fig. 2) and that subsequent fixation at 1 then serves to establish the two-dimensional fixation.

Since alcohols of such different structure as diglycerides, benzyl, cyanomethyl or fluoroethyl all lead to active substrates[1], and the aliphatic chain on the fatty acid side need not be present, it is clear that lipophilic binding cannot be very important in the formation of the enzyme–substrate complex. If to a chemist it seems surprising that with fixation only at 1, 2 and 3 (Fig. 2) a structural feature of R as far away as a $\Delta^{5,6}$ double bond should still exert hindrance, although the fatty acid chain is not bound, it must be remembered that the reaction probably takes place in a valley or trough on the enzyme and that, apart from the requirements of bonding, the entire substrate has to be spatially accommodated. It may help to visualize that the group —O—(C=O)—R is planar and that therefore with bonding at —O—, C, and O= the group R will extend horizontally along the plane of bonding if not bent away from carbon chain link 3 onward.

*Resistance of marine polyenoic acids*

BOTTINO *et al.*[8] have discussed the possibility that the resistance of certain all-*cis*-polyenoic acids may result from a folding of the acids in such a way that the

terminal methyl group approaches the carboxyl group. Such a hypothesis is not supported by our data. The addition of further *cis*-double bonds after the carboxyl-proximal double bond does not further increase the resistance. Furthermore, an acid with maximal and rigid refolding, *cis*-2-octenoic acid, is not more resistant than two similar acids in which the chain points straightly away from the carboxyl group, *trans*-2-octenoic and 2-octynoic acid.

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