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EFFECT OF SURFACE PRESSURE ON THE HYDROLYSIS OF ESTER MONOLAYERS BY PANCREATIC LIPASE

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SUMMARY

A Langmuir trough was converted into a fully automatic recording bidimensional barostat which was used for investigating in detail the effect of surface pressure on the digestion of various ester monolayers by pure pancreatic lipase (glycerol-ester hydrolase, EC 3.1.1.3).

When the substrate was 1,3-dihexanoyl 2-butylether glycerol or trihexanoyl glycerol, lipase was found to be entirely inactive in the lower pressure range and still poorly active until a pressure of 10 dynes·cm^{-1}. For higher pressure values, the activity rose abruptly, passed a sharp maximum and then decreased until film collapse. Maximal activity towards both glyceride substrates and also towards didecanoyl glycerol previously used by Olive and Dervichian was not attained for the same surface density, molecular area or area per fatty chain of the substrate, but for nearly the same film pressure. With the monoester *p*-chlorobenzyl decanoate film collapse for pressures not exceeding 10 dynes·cm^{-1} prevented the appearance of a large lipase activity. This activity, however, could also be shown to increase with pressure.

Among the several interpretations which may be offered for this pressure effect one supposes that lipase, upon adsorption at the interface, acquires a functional conformation for intermediary values of the free interfacial energy (and consequently of the film pressure). Lower and higher values would lead to inactive forms due to denaturation or insufficient conformational variations of the enzyme molecule.

INTRODUCTION

Lipases (glycerol-ester hydrolase, EC 3.1.1.3) are known to hydrolyze rapidly aggregates of ester molecules (emulsified particles[1] or micelles[2]) in an aqueous medium. The action of the pancreatic enzyme on long-chain triglyceride emulsions has been shown some years ago to be controlled by the concentration of the interface (interfacial area per unit volume of emulsion) separating the insoluble particles from water[3]. All other things being equal, this value is directly related to the concentration of the

substrate molecules situated at the interface and consequently available to the water-soluble enzyme.

The advantages of monolayers over emulsions for investigating the interactions of insoluble substrates with lipolytic enzymes have often been emphasized[4,5]. In addition to the fact already mentioned that the number of available substrate molecules in emulsions depends on the size of the particles, these emulsions often require stabilization by an emulsifier which affects the interface in an ill-defined manner. A monolayer, which is stable in the absence of any added tensioactive compound and in which all substrate molecules are in contact with the solvent containing the enzyme, constitutes a simpler system.

Moreover, by varying the surface pressure of the film, it is possible to modify a number of important parameters such as the surface density (number of substrate molecules per unit surface of the film) and its reciprocal value, the molecular area (area occupied by one substrate molecule in the monolayer), the general orientation of the film molecules with respect to each other and to the interface, the free energy of the surface, the tendency of the film molecules to form bidimensional aggregates and finally the structure of water in a layer situated immediately under the film. For all these reasons, important conclusions can be expected to emerge from a careful study of the pressure dependence of the enzyme-catalyzed film digestion.

The rate at which a substrate monolayer is enzymatically digested at any predetermined pressure can most conveniently be measured by the method of Dervichian[6]. Olive and Dervichian[7] recently investigated with the aid of this method the hydrolysis of didecanoyl glycerol monolayers by an exocellular lipase from the mold *Rhizopus arrhizus*. An interesting finding was that the reaction rate was very strongly affected by the pressure exerted on the film.

In the course of the present study, some difficulties were encountered with didecanoyl glycerol, probably because of the existence of an equilibrium between two isomeric forms (1,2 (or α, β) and 1,3 (or α, α')) of the diglyceride. These forms are known not to be equivalent for lipase[8]. Therefore, a stable triglyceride (trihexanoyl glycerol) and a diglyceride (1,3-dihexanoyl, 2-butylether glycerol) in which the internal position was permanently blocked by an ether linkage, were preferred. A monoalcohol ester (*p*-chlorobenzyl decanoate) was also employed for the purpose of comparison. With these three compounds, the striking dependence of lipase activity on film pressure was confirmed. Like didecanoyl glycerol, the two 3-chain substrates gave activity–pressure plots with a sharp maximum corresponding to a pressure value of 23 dynes$\cdot$cm^{-1}. *p*-Chlorobenzyl decanoate films turned out to be unstable for pressures beyond 10 dynes$\cdot$cm^{-1}, so that high rate values were never attained with this monoester, despite the known ability of similar compounds to be good substrates for lipase when emulsified in the presence of bile salts[9].

MATERIALS AND METHODS

(1) Lipase preparations

Pancreatic lipase was purified from defatted porcine pancreas powder according to Verger *et al.*[10]. The preparations, which were not passed through CM-cellulose, contained the two L_A and L_B forms of the enzyme. They also contained about 30% of the saturating amount of colipase[11]. Their specific activity at pH 9.0 (μmoles of

fatty acid liberated per min per mg protein) in the test using an olive oil emulsion stabilized by arabic gum[1] amounted to 3800. Stock solutions (1 mg/ml) of the enzyme in a 5 mM Tris–HCl buffer (pH 8.0) containing 0.15 M NaCl were kept at 4 °C and diluted before use to the desired concentration.

(2) Substrates

p-Chlorobenzyl decanoate was prepared by condensation of *p*- chlorobenzyl alcohol with distilled decanoyl chloride in dry chloroform–pyridine. After crystallization from 96% ethanol, the compound was found to be pure by thin layer chromatography on Silica gel G in a benzene–acetic acid (150:2, v/v) system.

The substrate 1,3-dihexanoyl 2-butyl ether glycerol was synthesized from pure 1,3-benzylidene glycerol[12] which was first converted into its sodium glycerolate derivative by metallic sodium in dry xylene and then etherified at position 2 with butyl bromide[13]. The 2-butyl ether glycerol resulting from removal of the benzylidene group by acid treatment[14] was dissolved in hexane and the solution was purified by passage through a silicic acid column. After 2 successive washings of the column by a 40:60 and 20:80 (v/v) hexane–diethyl ether mixture, the compound was eluted by pure diethyl ether, a 90:10 (v/v) diethyl ether–methanol mixture and absolute methanol. Finally, positions 1 and 3 in the butylether were esterified by pure hexanoyl chloride in anhydrous chloroform–pyridine. The resulting product was dissolved in hexane and this solution was purified by passage through a Florisil column equilibrated with a 85:15 (v/v) hexane–diethyl ether mixture. The purity of the preparation was checked by thin layer chromatography on Silica gel G in a 55:40:5:0.2 (by vol.) diethyl ether–hexane–chloroform–acetic acid system.

Trihexanoyl glycerol was prepared by condensation of glycerol with 3.6 moles/mole of pure hexanoyl chloride in chloroform in the presence of an excess of dry pyridine. After removal of hexanoic acid and partial glycerides by passage through active carbon and Florisil, the purity of the compound was checked by thin layer chromatography on Silica gel G in a 50:50:1 (v/v) hexane–diethyl ether–acetic acid system. Dihexanoyl glycerol was obtained from pure trihexanoyl glycerol by digestion with pancreatic lipase. The diglyceride was isolated and purified by preparative thin layer chromatography on Silica gel G in a 70:30:2 (v/v) hexane–diethyl ether–formic acid system.

(3) Description of the apparatus

The Langmuir trough and accessories were built in Professor Dervichian's workshop (Institut Pasteur, Paris). They were identical to those used and already described in detail by this author[6]. The trough was essentially composed of: (a) A 100 cm × 20 cm × 3 cm plexiglass cuvette placed in a cabinet thermostated at 22.5 °C by circulating water. The liquid in the cuvette was also thermostated at the same temperature. (b) A floating plastic frame limiting the film. (c) A plastic strip acting as a two dimensional piston (the mobile barrier) placed transversally on the frame and driven longitudinally by an electric synchroneous motor at a constant rate which could vary from 2.7 to 85.8 $cm \cdot min^{-1}$. Forward displacements of the mobile barrier were used for obtaining film compression isotherms, for compressing the film to any predetermined pressure value and finally for maintaining in the film a constant pressure in the course of an enzymatic digestion. (d) A two-dimensional manometer

with the aid of which the film pressure was continuously known and recorded.

In addition, a significant improvement due to Dr. G. Benzonana in this laboratory was to include in the system an electronic monitor by which the apparatus was converted into a fully automatic two-dimensional barostat. This monitor induced the forward displacement of the mobile barrier just necessary to compensate for any fall of the film pressure under a predetermined value. It also insured the recording of the film surface (or length) as a function of time. Since the film surface under a constant pressure was proportional to the number of molecules remaining in the film at any moment, this recording could be assumed to provide a direct access to the kinetic course of the reaction. The fact that the monitor and the driving motor responded within less than half a s to pressure variations as low as 0.02 dyne·cm^{-1} was considered as very satisfactory.

(4) Description of the assays

The apparatus described above was used, either in the absence of enzyme to determine the properties of the film and of its components, or in the presence of enzyme to follow the digestion of the molecules composing the film. In both cases, the trough was filled with 4 l of a 5 mM Tris buffer (pH 8.0) prepared with quartz-bidistilled water and containing 0.15 M NaCl. Dust and any other solid impurities floating on the surface were carefully removed by suction and the absence of tensio-active contaminants was checked by several compressions which should induce no detectable pressure variations.

For assays without enzyme, the film was spread by addition on the aqueous surface, with the aid of an Agla microsyringe, of some droplets of a 1 mg/ml hexane solution of the investigated compound. The mobile barrier was displaced at a constant rate and the pressure variation was recorded.

For digestion assays, a known volume of lipase stock solution was measured with a micropipette and usually diluted to 10 ml with the buffer. This volume was never lower than 50 μl in order to insure a good accuracy. If required, the stock solution was diluted in more than 10 ml or the 10 ml dilution was diluted a second time before use. Then, the dilution was injected with the aid of a syringe into the buffer situated between the two antidiffusion barriers of the Langmuir trough (about 2 l). The syringe needle was bent so that the tip, which had been flattened, could be maintained horizontal during injection while its position and direction were continuously changed. In spite of the presence on the liquid surface of the plastic frame which did not permit a vigorous stirring of the mixture, a satisfactory dispersion of lipase throughout the buffer was attained, as is proved by the two following observations: (a) A number of assays with a given substrate under the same experimental conditions consistently led to identical results; (b) a strict proportionality was always observed between the concentration of lipase in the subphase and the digestion rate constant (see later).

In a second step, the monitor was set up to the desired pressure value π and a slight excess of the substrate solution in hexane was placed on the surface of the cuvette. The pressure at once began to fall under the influence of lipase and it was later controlled automatically by the monitor through suitable displacements of the mobile barrier. L *vs* time plots were recorded until L dropped below 25% of L_0. In all cases, the initial period necessary for hexane evaporation and pressure stabi-

lization was short when compared to that during which the reaction kinetics were recorded.

(5) Kinetic treatment of the data

A reaction taking place in a monolayer under a constant pressure induces, like any other reaction, a progressive decrease of the number of substrate molecules. However, this decrease does not result here in a parallel drop of the substrate concentration, but rather in a decreasing film surface. It is, therefore, possible to relate the reaction rate to the "interface or surface concentration" (interface or surface area in one unit volume of any heterogeneous system) defined by Benzonana and Desnuelle in the case of emulsions[3]. Since the volume of the aqueous subphase containing the enzyme is constant during the assays, the surface concentration is proportional to the surface of the film and consequently to its length L. In fact, linear log L/L_0 *vs* time plots were obtained, as shown by Fig. 1, except in a few assays carried out at very high pressure. Most of the time, Eqn 1 giving a first value k' for the rate constant was verified.

$$\log L/L_0 = k't \tag{1}$$

Another constant, $k = k'/E_0$, independent from the enzyme concentration was also calculated and used for the pressure–activity plots illustrated by Figs 5–7.

It is noteworthy that the above treatment takes into account the variations of the film surface rather than those of the number of substrate molecules as it should normally do. However, rate constants, not rates, were measured so that no correction was necessary when results obtained at different pressures and with different substrates were compared.

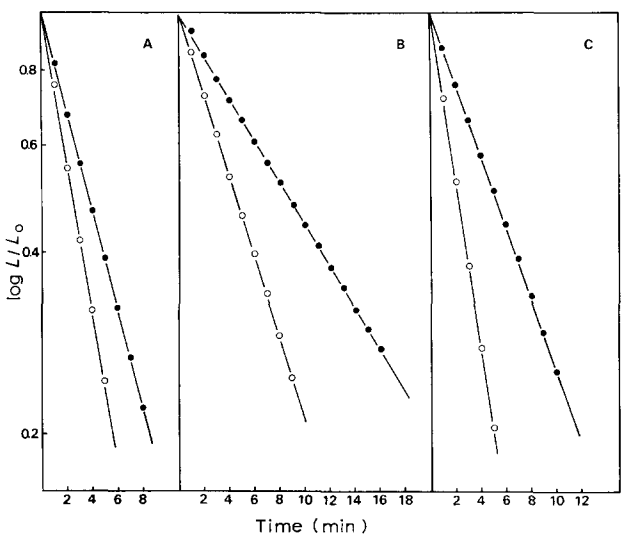


Fig. 1. Typical linear log $L/L_0 = f(t)$ plots with *p*-chlorobenzyl decanoate (A), 1,3-dihexanoyl 2-butylether glycerol (B) and trihexanoyl glycerol (C). The lipase concentration in the subphase was approx. $17 \cdot 10^{-10}$ M for A and $1.5 \cdot 10^{-10}$ M for B and C. Assays at 2 different film pressures were performed with each substrate. The pressure values were for the upper (●—●) and lower (○—○) curves, respectively: A, 4.16 and 7.24; B, 12.38 and 15.13; C, 12.38 and 17.88 dynes·cm^{-1}.

According to Benzonana and Desnuelle[3], the lipase-catalyzed hydrolysis of long-chain triglyceride emulsions obeys the Michaelis–Menten rule. An increase of the interface concentration induces enzyme saturation in the sense that all the enzyme molecules initially dissolved in water are finally adsorbed by the interface. Hence, the two kinetic parameters k_{cat} and K_m of the reaction are experimentally accessible. By contrast, the following observation shows that the amount of lipase bound to the monolayer can for all practical purposes be considered as negligible when compared to that remaining in the subphase: A triglyceride film was spread over a lipase-containing subphase, partly digested, removed by suction and replaced by another film which was digested under exactly the same conditions. The second digestion was not appreciably slower than the first. Since the same observation also holds for pancreatic phospholipase A (ref. 15), the monolayer technique does not appear to give access to the kinetic parameters k_{cat} and K_m, but only to their ratio as shown by Eqn 2 written under the very likely assumption that $(S) \ll K_m$.

$$v = \frac{k_{cat} \cdot (E_0) \cdot (S)}{K_m + (S)} = \frac{k_{cat}}{K_m} \cdot (E_0) \cdot (S) \qquad (2)$$

(6) Substrate insolubility and product desorption

It was pointed out earlier in this section that correct results could not be obtained by the monolayer technique unless the substrate molecules composing the monolayer did not leave the surface spontaneously even at high pressure and the rate at which products diffuse into the subphase was much higher than the reaction rate. A first observation showing that no molecule leakage from the film occurred during our assays was that compression isotherms were never affected by a variation of the speed at which the mobile barrier was displaced. Moreover, no detectable pressure fall could be discerned when *p*-chlorobenzyl decanoate and trihexanoyl glycerol films were maintained for an extended period of time at pressures immediately below that inducing collapse. With 1,3-dihexanoyl 2-butyl ether glycerol, the resulting pressure decrease did not exceed 0.01% of the lowest observed digestion rate.

Problems related to product desorption were also carefully investigated. The most convincing proof that desorption was never rate-limiting in our assays was derived from the strict linearity of the log L/L_0 *vs* time and k' *vs* E_0 plots reproduced in Figs 1–3. Other experimental proofs were obtained by observing directly the behavior of the products at the interface. Decanoic acid, previously reported to have surface properties in the acidic pH range[15], was mixed with an equivalent amount of *p*-chlorobenzyl decanoate. Compression of the mixed film spread over a subphase at pH 8.0 resulted in an almost instantaneous pressure decrease towards the value expected for the ester alone. The shorter hexanoic acid should behave in the same way. Moreover, no stable films could be formed with dihexanoyl glycerol, the other digestion product of trihexanoyl glycerol. The compound 1-hexanoyl 2-butyl ether glycerol arising from the digestion of dihexanoyl 2-butyl ether and containing 2 carbon atoms less was not synthesized. But it could be expected to be still more soluble than the first.

The strict proportionality existing between k' and E_0 in Figs 2 and 3 also confirmed that the monolayer technique was well suited, as already pointed out by other authors[16,17], for the quantitative determination of lipase activity.

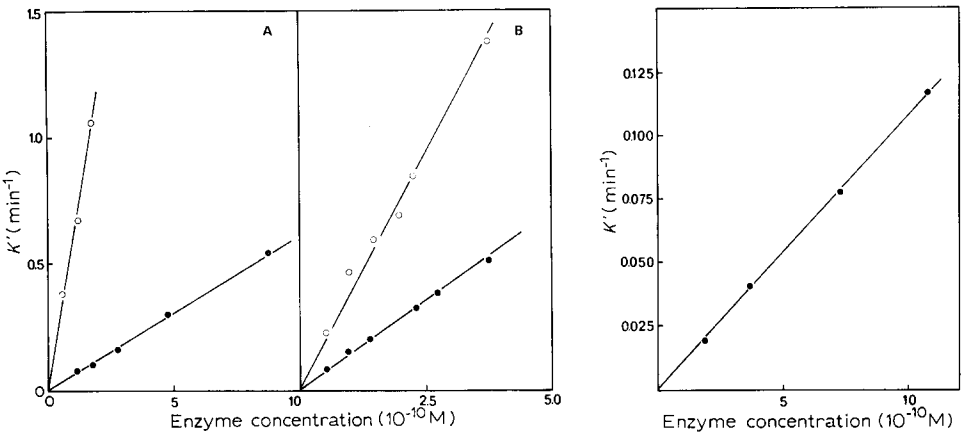


Fig. 2. k' plots as a function of E_0. The pressure values were for the upper (○—○) and lower (●—●) curves, respectively, 23.40 and 12.38 dynes·cm^{-1} for A (1,3-dihexanoyl 2-butyl ether glycerol); 17.88 and 13.76 dynes·cm^{-1} for B (trihexanoyl glycerol).

Fig. 3. k' plots as a function of E_0. Case of *p*-chlorobenzyl decanoate. Pressure, 4.16 dynes·cm^{-1}.

(7) Stirring of the subphase

The solution of lipase in the aqueous subphase was carefully stirred before film spreading in order to avoid gross heterogeneity. But, stirring was discontinued during digestion mainly because perfectly linear log $L/L_0 = f(t)$ plots were obtained without stirring. It must be stressed, however, that a 4-fold acceleration by stirring of the hydrolysis of phospholipid monolayers by pancreatic phospholipase was recently reported[15]. The origin of this puzzling effect is still unknown.

RESULTS

(1) Compression curves

The molecular area and its reciprocal value, the surface density of the substrate, can be calculated when the film surface, the film weight and the molecular weight of the substrate are known. Pressure–molecular area plots, usually designated compression curves or compression isotherms, are reproduced in Fig. 4 for the 3 ester substrates used in the present assays. The two 3-chain glycerol derivatives are seen to behave similarly upon compression. Below 5 dynes·cm^{-1}, the molecular area varies considerably with pressure, indicating that, in this range, the films have properties similar to that of a gas in which the molecules are relatively far and independent from each other (vapor-expanded state). Above 5 dynes·cm^{-1}, the pressure increments necessary for further reduction of the surface become progressively larger until the film collapses. This collapse occurs in both cases at approx. 24 dynes·cm^{-1}.

By contrast, the monoester film never appears to be in a vapor-expanded state and its collapse pressure does not exceed 10 dynes·cm^{-1}.

(2) Dependence of lipase activity on film pressure

The pressure dependence of the rate constants of the lipase-catalyzed film hydrolysis is illustrated by Figs 5–7. Each point in these figures represents the average

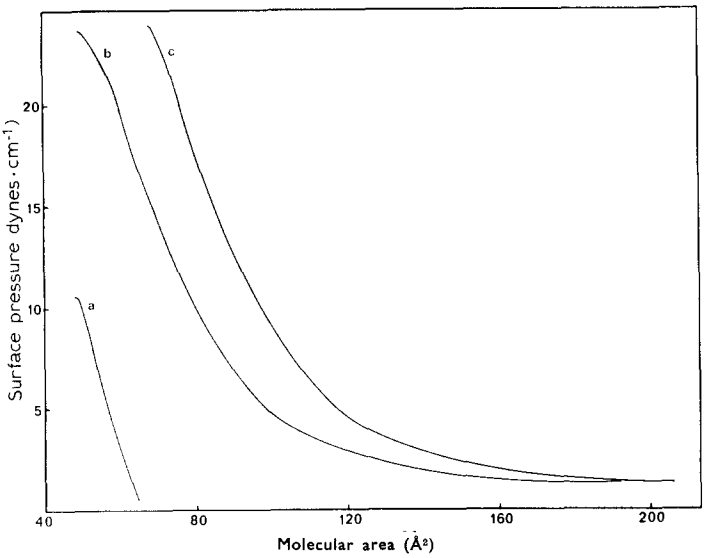


Fig. 4. Pressure–molecular area plots with *p*-chlorobenzyl decanoate (a), 1,3-dihexanoyl 2-butyl ether glycerol (b) and trihexanoyl glycerol (c). The mobile barrier was displaced at a constant rate of 5.35 cm per min for *p*-chlorobenzyl decanoate and 10.7 cm per min for the two other substrates

of at least 5 independent assays. The curves related to the 3-chain glycerol derivatives (Figs 5 and 6) are seen to be very similar again. For pressures below 5–10 dynes·cm^{-1}, lipase is inactive or very weakly active. Above 10 dynes·cm^{-1}, activity increases more and more abruptly to attain a sharp maximum corresponding to a pressure of approx. 23 dynes·cm^{-1}. The maximum is defined by a single point in Fig. 6. But this point is certainly significant since, as already reported above, it represents the average of 5 assays. The maximal fluctuation range between assays performed under the same conditions did not exceed 10–12%.

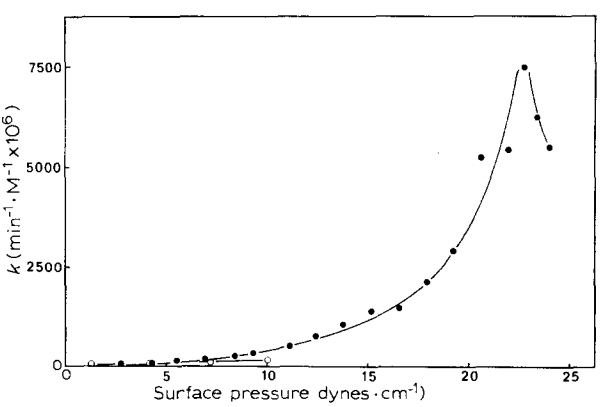
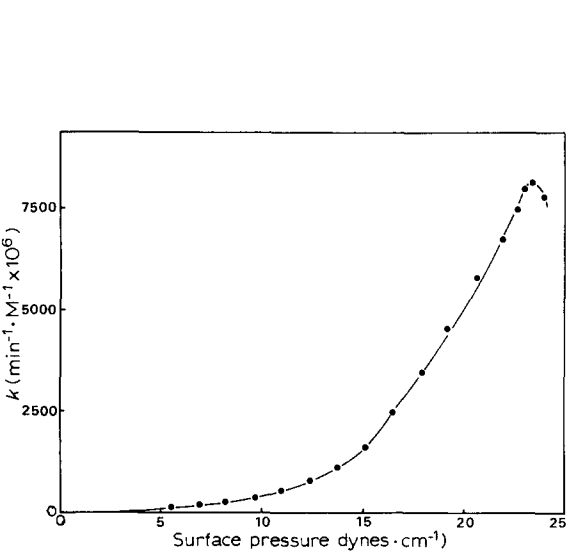


Fig. 5. Pressure dependence of film digestion. Case of 1,3-dihexanoyl 2-butyl ether glycerol. For each assay, the film pressure was set to the desired value and the digestion rate at this pressure was measured as described in Materials and Methods.

The molecular area corresponding to this optimal pressure is 54 Å^2 with 1,3-dihexanoyl 2-butylether glycerol and 71 Å^2 with trihexanoyl glycerol. The surface density values are, respectively, 1.84 and $1.42 \cdot 10^{14}$ molecules per cm^2.

The ordinates in Fig. 7 related to the monoester *p*-chlorobenzyl decanoate are much larger than those in Figs 5 and 6, in order to compensate for a lower lipase activity. However, the two fundamental facts already stressed in the case of the glycerol derivatives, namely very weak lipase activity at low pressures and subsequent steep activity increase can be confirmed with the monoester. Apparently, high lipase activities never develop with the monoester because of early film collapse.



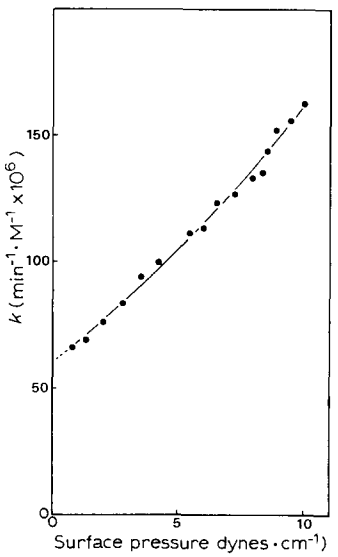


Fig. 6. Pressure dependence of film digestion. Case of trihexanoyl glycerol. Same legend as for Fig. 5.

Fig. 7. Pressure dependence of film digestion. Case of *p*-chlorobenzyl decanoate. Same legend as for Fig. 5.

DISCUSSION

Early assays[4,5,18,19] had already suggested in the past that the pressure exerted on a phospholipid monolayer affected the rate of its digestion by phospholipase. More recently, however, the mode of action of lipase on glyceride monolayers[16,17] was investigated at constant film area and consequently varying pressure. The fact that apparently good results were obtained in this manner might have indicated that surface pressure was less critical for lipase than for phospholipase. But, when satisfactory techniques for maintaining a constant pressure throughout the assays became available[6,15], the rate at which both glyceride and phospholipid monolayers were digested by the corresponding enzymes was found to be strongly pressure dependent[7,15].

In the course of the present study, assays were extended to pure pancreatic lipase, two fatty glycerol derivatives and the fatty ester of a monoalcohol. The activity–pressure plots related to the two glycerol derivatives were found to be almost

identical and also to be very similar to that previously obtained with didecanoyl glycerol by Olive and Dervichian[7]. All showed a considerable activity increase in the 10–20 dynes·cm^{-1} range, a sharp maximum at 20–23 dynes·cm^{-1} and an abrupt activity drop for higher pressures. Additional and probably the most important information given by Figs 5 and 6 was that lipase turned out to be completely inactive at very low pressure and to display only a very weak activity until 10 dynes·cm^{-1}. Moreover, the subsequent activity increase had a pronounced upwards curvature and the corresponding variation was perfectly smooth. These observations will undoubtly facilitate the final interpretation of the data.

Another notable finding was that the maximal lipase activity towards the 3 glycerol derivatives investigated so far was attained for the same, or nearly the same, surface pressure (23 dynes·cm^{-1} in our assays (see Figs 5 and 6); 20 dynes·cm^{-1} in ref. 7). By contrast, Fig. 8 indicates that this maximal activity corresponds to widely different surface density values of the substrate (1.42 and 1.84·10^{14} molecules per cm^2 for our substrates; 1.60·10^{14} for didecanoyl glycerol[7]). The molecular area (54 and 71 Å^2 in Fig. 8; 60 Å^2 in ref. 7 and the area per fatty chain are also different. The conclusion at this point is that the activity of lipase on a substrate monolayer is not controlled, as it may have been expected to be, by simple factors such as the two dimensional concentration of the substrate in the film (surface density), but rather by another parameter (or other parameters), closely related to surface pressure. The identification of this parameter would undoubtly lead to a better understanding of the known requirement of lipase (and phospholipase as well[20]) for aggregated substrates.

At present, experiments are being carried out in our laboratory to check the validity of the following hypothesis: Pancreatic lipase has been assumed for a number of years to be inactive when dissolved in water or an aqueous buffer, and to be somehow activated upon adsorption at an hydrophobic interface[1]. Several proteins are known to adsorb at surfaces or interfaces and there to undergo conformational modi-

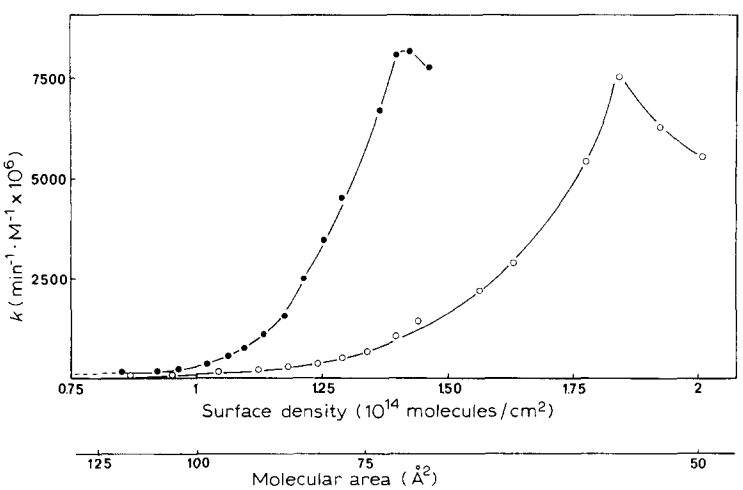


Fig. 8. Lipase activity plot as a function of surface density or molecular area. ○—○, 1,3-dihexanoyl 2-butyl ether glycerol; ●—●, trihexanoyl glycerol.

fications due to the free energy of surface or free interfacial energy[21–25]. The characteristic property of lipase would be that, when conditions at the interface are favorable, a functional active site is created in the adsorbed enzyme, as it is created for instance in chymotrypsin after cleavage of a peptide bond in the corresponding zymogen. Now, since the free energy of surface and surface pressure vary inversely, the bell-shaped curves in Figs 5 and 6 would be due to the existence of reversible transconformations of lipase molecules leading to at least 3 states: (1) a native and inactive state favored by high film pressure (low energy), (2) a slightly modified and active state favored by intermediary values of pressure and energy and (3) a "denatured" and again inactive state favored by low pressure (high energy). This hypothesis is already supported by a recent report[26] according to which lipase is inactivated, probably by denaturation, either at air–water interfaces or in systems containing an hydrocarbon–water interface where the free interfacial energy can be expected to be especially high. This inactivation is largely prevented by addition of bile salts lowering the free interfacial energy. In the same respect, it is noteworthy that *p*-chlorobenzyl decanoate emulsified in water is a poor substrate for lipase (Sémériva, M., unpublished experiments) despite the activating effect exerted by the *p*-chlorobenzyl group on the ester bond. The same compound is a good substrate, however, when the emulsions are prepared in the presence of bile salts.

Other assumptions worthy of consideration are that the pressure-dependent parameter controlling the rate is the orientation of the substrate molecules at the interface[15], the formation in the film of molecular aggregates equivalent to micelles[2] and on which lipase would act preferentially, the structure of water in the vicinity of an hydrophobic interface.

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