Hydrolysis of Mixed Monomolecular Films of Triglyceride/Lecithin by Pancreatic Lipase*

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The main purpose of this study was to describe the influence of lecithin upon lipolysis of mixed monomolecular films of trioctanoylglycerol/didodecanoylphosphatidylcholine by pancreatic lipase in order to mimic some physiological situations. The quantity of enzyme adsorbed to the interface was simultaneously determined using 5-thio-2-nitro[¹⁴C]benzoyl lipase. Lipolytic activity was enhanced 3- to 4-fold in the presence of colipase, an effect which is attributed to increased enzyme turnover number. When a pure triglyceride film was progressively diluted with lecithin, the minimum specific activity of lipase exhibited a bell-shaped curve: a mixed film containing only 20% trioctanoylglycerol was hydrolyzed at the same rate as a monolayer of pure triglyceride.

For the sake of simplicity, most studies of model interfaces have involved only one type of lipid and/or protein. In the same context, most studies of lipolytic enzyme kinetics were primarily done in vitro with pure lipids as substrates (1). In reality, however, virtually all biological interfaces are composed of complex mixtures of lipids and proteins. Thus, intestinal lipolysis, blood chylomicrons and the hydrolysis of lipoproteins, as well as intracellular lipolytic activities, all involve the simultaneous participation of several classes of lipids, e.g., glycerides, phospholipids, cholesterol derivatives, bile salts, etc. Studies of membrane phospholipid hydrolysis by phospholipases (2) also present this characteristic of lipid complexity. The main purpose of this study was to describe the influence of lecithin upon lipolysis of mixed monomolecular films of trioctanoylglycerol/didodecanoylphosphatidylcholine by pancreatic lipase in order to mimic the above physiological situation.

We adopted the monolayer technique for the study of the mode of action of lipolytic enzymes on mixed lipid interfaces. This method offers the unique and fundamental advantage of allowing one to control the “quality of the interface,” which is determined by molecular orientation, molecular and charge specificities, water structure, fluidity, and lipid composition (1, 3). We utilized a radiolabeled pancreatic lipase to determine the influence of film composition on enzyme penetration and/or turnover at the interface. Only mixed trioctanoylglycerol/didodecanoylphosphatidylcholine films were used in the present study. Of special interest to us was the relatively recent concept of Scow et al. (4, 5), according to which triglyceride hydrolysis may be considered the first step in lateral flow lipid transport in cell membranes.

The main conclusion drawn from this work is that considerable activation or inhibition may result as a function of lipid composition, lipid packing, and surface defects in mixed films.

EXPERIMENTAL PROCEDURES

Enzymes—The enzymes, film constituents, and water were of the same quality as described in prior studies (3, 6, 7). 5-Thio-2-nitro[¹⁴C]benzoyl lipase was obtained as described (8) and had a specific radioactivity of 3.92·10⁴ dpm/mg of protein. The mixture of porcine pancreatic colipases 1 + II was prepared in our laboratory (9).

The subphase of the reaction compartment was prepared by adding a known weight of chloroform. Lipid purity was verified by thin layer chromatography; samples were spread with an Agla micrometer syringe unit (Burroughs-Wellcome). Solutions of known concentration of trioctanoylglycerol and didodecanoyl-lecithin in chloroform were mixed in different proportions. Mixed films were obtained by spreading these solutions.

Lipid compression isotherms were obtained with the reservoir compartment of the zero order trough (10) by incremental compressions of 1 dyne cm⁻¹ min⁻¹.

Monitoring Reaction Rates—The surface barrier method used has been described elsewhere (10). The zero order trough was composed of a reservoir 17.6 X 28.4 cm and a reaction compartment containing the enzyme (total volume 210 ml; total surface, 123.7 cm²). All experiments were performed at 25 ± 0.5°C. The following standard buffer was used in the assays: 10 mm Tris/acetate, pH 6.0, 0.1 m NaCl, 21 mm CaCl₂, 1 mm EDTA. The buffer was filtered through a charcoal column and pH was adjusted immediately prior to use. The pH value of 6.0 was chosen in order to reduce the rate of lipolysis thus allowing addition of larger amounts of radiolabeled enzyme to be adsorbed to the monomolecular film facilitating its detection. Before each experiment, the trough was cleaned with ethanol, rinsed several times with tap water until the Teflon surface was no longer retaining drops of water and then rinsed with distilled water. Unlike previous experiments, no detergent solution was employed.

Measurement of Hydrolysis of Mixed Lipid Monolayers—A Teflon barrier was placed transversely over the small channel of the zero order trough in order to block surface communication between the reservoir and reaction compartment. Surface pressure was initially determined by placing the platinum plate in the reaction compartment where the mixed film was spread at the desired pressure. Surface pressure was then measured after switching the platinum plate to the reservoir compartment where the pure substrate film was subsequently spread. The surface pressure was then adjusted to the value in the reaction compartment by displacement of the mobile barrier. The subphase of the reaction compartment was agitated with two magnetic stirrers turning at 750 rpm. The barrier between the two compartments was then removed in order to allow communication of the two films. Pressure change during these operations did not exceed 0.25 dyne/cm². The enzyme was then injected into the reaction compartment and kinetics were recorded as described (10) The principle of the method is schematically shown in Fig. 1.

Footnotes:

* This is Paper VI in a series on enzyme reactions in a membrane model. Paper V is identified as Ref. 8. These results were presented during the EMBO-Workshop on Structure and Function of (phospho)lipases held in Cassis, France from October 15-19, 1978. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Lipase Hydrolysis of Mixed Monomolecular Films

Recovery of Films, Determination of Radioactivity, and Calculation of the Minimal Specific Activity—A method for the quantitative recovery of a surface film was used (7). Fifteen minutes after enzyme injection, a surface of 120 cm² was collected in a scintillation vial in a volume not exceeding 3 ml. The same volume of subphase was added to another vial. A liquid scintillator for aqueous samples (Instagel, Packard Instruments) was used in the clear liquid phase. Radioactivity was determined with a Packard 2450 liquid scintillation spectrometer. The difference in counts between the two vials represents the radioactivity of the film. From this value one can calculate the interfacial excess of enzyme expressed in milligrams/cm². Because the monolayer technique allows the measurement of the enzyme velocity expressed in molecules/cm²/min and the interfacial excess of enzyme in milligrams/cm², it is easy to obtain for each lipid mixture a value of a minimal specific activity of the pancreatic lipase expressed as usual in micromoles/min/mg. We use the term minimal specific activity because the surface radioactivity measured represents not only those enzyme molecules directly involved in the catalysis but also an unknown amount of protein present close to the monolayer. These enzyme molecules need not be necessarily involved in the enzymatic hydrolysis of the film.

Possibilities of Film Segregation—It was macroscopically verified that no lipid segregation occurred in the mixed lipid film over the reaction compartment during hydrolysis of one of the two constituents. An equimolar mixture of trioctanoylglycerol and [¹⁴C]lecithin from rat liver microsomes (11) was spread over the reaction compartment and pure trioctanoylglycerol was spread over the reservoir at a surface pressure of 20 dynes/cm, as described above. After injecting 0.45 µg of pancreatic lipase into the aggregated subphase of the reaction compartment, kinetics were followed for 15 min with the barostat method (10). At the end of this period, it was found that 90% of the triglyceride present in the reservoir had been hydrolyzed. A Teflon barrier was first placed transversely across the small channel of the zero order trough to block surface communication between the two compartments. A second Teflon barrier was then immediately placed longitudinally across the reaction compartment to divide its surface into two equal portions. The two half-surfaces were then separately collected in scintillation vials as described above. Recovery of [¹⁴C]-lecithin was 100% and the ratio of counts in the two half-surfaces was 1:07.

Enzyme Proportionality—One criterion for the study of enzyme reactions with the monomolecular film method involves the verification of proportionality between rate constants and enzyme concentration. Reaction velocity as a function of enzyme concentration was a straight line which, for unknown reasons, did not pass through the origin (10); we observed a similar phenomenon in the present study (see Table I).

As shown in Table I, lipase activity with pure trioctanoylglycerol and mixed films of trioctanoylglycerol/didodecanoylphosphatidylcholine increased 2.7-fold when enzyme concentration was increased 4-fold. Since interfacial enzyme concentration also increased 2.7-fold, minimal specific activity remained nearly constant under identical experimental conditions. The apparent lack of strict linear proportionality may thus be attributed to only the adsorption step and not to subsequent catalytic steps, including product desorption from the interface.
The influence of colipase on the rate of enzymatic hydrolysis of trioleoylglycerol by $^{[14]C}$TNB-lipase (5.5 pg) as a function of the proportion of trioleoylglycerol in mixed films of trioleoylglycerol/dodecanoylphosphatidylcholine. Experimental conditions as described in legends to Figs. 3 and 4. Standard errors are shown by bars.

Properties of Mixed Lecithin/Triglyceride Films—It was shown by Desnuelle et al. (16) that a crude extract of unsaturated triglycerides led to the condensation of a phospholipid film. These authors also observed strong phospholipid-triglyceride interactions at 30 and 70% phospholipid in the mixed films. It was shown by Torosian and Lemberger (17), however, that plots of extrapolated mean cross-sectional area versus mole fraction in the lecithin-triglyceride system were apparently linear, indicating an ideal two-dimensional liquid. The present data, which confirm those of Desnuelle et al. (16), clearly show that there is a continuous shift in the apparent collapse pressure of the different dodecanoylphosphatidylcholine/trioleoylglycerol mixtures. According to Gaines (18), this type of behavior is to be expected of two miscible lipid components in monolayers. Significant quantities of the triglyceride remain in the interfacial film as a result of this miscibility, even at a surface pressure higher than the collapse pressure of pure trioleoylglycerol (20 dynes/cm) (cf., Fig. 2). The same conclusion was reached with mixed trioleoylglycerol/egg lecithin films in an argon atmosphere. Although the collapse pressure of a pure trioleoylglycerol film is 13 dynes/cm, it can be calculated that up to 22% of the film is occupied by the long-chain triglyceride at 30 dynes/cm (data not shown). This interfacial behavior enables us to say that most

Fig. 2 enables us to calculate that about 20% of the surface of the monolayer is occupied by triglyceride molecules at a pressure of 30 dynes/cm. This film composition is independent of the molar lecithin/triglyceride ratio in the original mixture.

Hydrolysis of Mixed Lecithin/Phospholipid Monomolecular Films by Pancreatic Lipase—Variations of reaction velocity and interfacial TNB-lipase concentration as a function of the molar fractions of trioleoylglycerol and didecanoylphosphatidylcholine are shown in Fig. 3. Maximal velocity is reached at a molar triglyceride fraction of 0.6 in the mixed film, while the interfacial TNB-lipase concentration decreases quasi linearly with increasing phospholipid molar fractions. It can also be seen that enzyme activity with a film of pure substrate can be doubled by progressively diluting the triglyceride film with phospholipid. We verified that unmodified pancreatic lipase exhibited a similar dependence of velocity on the molar fraction of trioleoylglycerol under conditions of optimal pH (pH 8.0 buffer). It is clear (Fig. 3) that plots of extrapolated minimal specific activity versus molar triglyceride fraction in mixed films of trioleoylglycerol/didecanoylphosphatidylcholine are bell-shaped curves, and that maximal activity of the enzyme is reached at a molar triglyceride fraction of 0.6 in the mixed film. These authors also observed strong phospholipid—triglyceride interactions at 30 and 70% phospholipid in the mixed films. It was shown by Torosian and Lemberger (17), however, that plots of extrapolated mean cross-sectional area versus mole fraction in the lecithin-triglyceride system were apparently linear, indicating an ideal two-dimensional liquid. The present data, which confirm those of Desnuelle et al. (16), clearly show that there is a continuous shift in the apparent collapse pressure of the different didecanoylphosphatidylcholine/trioleoylglycerol mixtures. According to Gaines (18), this type of behavior is to be expected of two miscible lipid components in monolayers. Significant quantities of the triglyceride remain in the interfacial film as a result of this miscibility, even at a surface pressure higher than the collapse pressure of pure trioleoylglycerol (20 dynes/cm) (cf., Fig. 2). The same conclusion was reached with mixed trioleoylglycerol/egg lecithin films in an argon atmosphere. Although the collapse pressure of a pure trioleoylglycerol film is 13 dynes/cm, it can be calculated that up to 22% of the film is occupied by the long-chain triglyceride at 30 dynes/cm (data not shown). This interfacial behavior enables us to say that most
natural lipase substrates (chylomicrons, lipoproteins, or alimentary fats) are probably mixed lipid films. Since a relatively large fraction of these interfaces is occupied by glyceride molecules, it is probable that the external monolayer surrounding the complex natural lipoprotein particles contains glycerides, as previously shown by chemical analyses (19, 20). This external glyceride pool could be in dynamic equilibrium with the remaining glyceride core. With this model, we can see that there is no absolute requirement for lipases to cross or hydrolyze the superficial shell in order to reach their substrate.

**Substrate Dilution**—The concept and principle of substrate dilution, which we believe is more correct than the term "surface dilution" (21), has been widely used in lipolytic enzyme kinetics (21-29). In order to "dilute" or "concentrate" a water-insoluble substrate (generally a lipid), the least complicated experimental approach, extensively used by Dennis and co-workers (21, 25, 28, 30), involves mixing the lipid substrate with a detergent or another lipid which is not a substrate for the enzyme.

It was observed by Dennis and his group that when the molar ratio of Triton X-100 to phospholipid exceeded a certain value, the rate of hydrolysis by phospholipase A2 decreased. It was suggested that this decrease resulted from surface dilution of the mixed micelles by inert detergent molecules. It has never been rigorously proven, however, that Triton X-100 is an inert spacer. Sundler et al. (28) recently purified a phospholipase C which specifically hydrolyzes phosphatidylinositol. With phospholipid as the substrate in mixed micelles with Triton X-100, they showed that the rate of catalysis was highly dependent on the detergent/phospholipid ratio, as previously reported by Dennis et al. (30) for other phospholipases. The decreased catalytic rates observed at high Triton/phospholipid ratios, however, were not attributed to substrate dilution on the micellar surface, since substrate dilution with phosphatidylcholine, either in mixed micelles (at a constant Triton/phospholipid ratio) or in bilayer vesicles, had no effect on catalysis (28). Depending on the enzyme and lipid used, however, more or less enzyme present in the interface could be bound to the substrate or to the dilutor, as a function of respective interfacial affinity constants. The interfacial enzyme-phosphatidylinositol complex is more easily dissociated by Triton molecules than by phosphatidylcholine, which explains the apparently contradictory results of Sundler et al. (28). As discussed by Verger and de Haas (1), some lipids or detergent in a mixed interface could behave as weak or strong competitive inhibitors or as pure inert substrate dilutors. When enzyme activity on such mixed micellar substrates is studied, it must be borne in mind that not only substrate density or substrate dilution is affected, but the "interfacial quality" of the surface is also changed (3), which can affect the ratio of free to adsorbed enzyme. In addition, in the case of mixed micelles with Triton X-100, where all the molecules are in principle readily accessible at the surface, it has been shown that different micellar types can exist (31); this renders the interpretation of the results even more difficult. In order to circumvent these difficulties inherent in bulk experiments, we used another method, in which mixed lipid monolayer studies were performed in such a way as to enable the adsorption and catalytic steps to be independently studied.

**Influence of Film Composition on Enzyme Penetration** It has been reported that lipid packing affects the quantity of lipolytic enzymes bound to interfaces (6, 8). Increasing the surface density of lipids usually leads to a decreased penetration of enzymes. It remained possible that nonspecific binding occurred at low surface pressure, with the accumulation of inactive protein at the surface (7). We showed (Fig. 3) that the amount of bound lipase decreases linearly with increasing proportions of lecithin in mixed triglyceride/lecithin films kept at constant pressure. This suggests that penetration of mixed films by pancreatic lipase shows a specificity for the triglyceride; i.e., enzyme penetration into an interface is affected by "interfacial quality." In addition, the film collection method (7) yields a reasonable and specific estimate of the quantity of bound enzyme.

An interesting finding was the effect of colipase on lipase binding to the interface (Fig. 4). Enzyme penetration of mixed films is different in the presence of the cofactor. Thus, lipase can bind to a film of pure lecithin at 12 dynes/cm only in the presence of colipase. This finding is to be compared with the observed increase of critical surface pressure from 23 to 30 dynes/cm, due to colipase which enables lipase to penetrate a film of 1,2-didodecanoylphosphatidylglycerol (8). It should be pointed out, however, that in the case of phosphatidylcholine films no enzymatic activity could be detected, even though lipase can penetrate the lecithin film in the presence of colipase. This lipid binding in the absence of detectable hydrolysis can be viewed as a physiological advantage, since Lairon et al. (32) reported that colipase is required for the formation of the lipase-bile lipoprotein complex (containing lecithin) and that bile lipids are needed to direct the adsorption of this lipolytic entity onto the emulsified substrate. In the context of this model, it can be seen that bile lipids must be resistant to lipolysis.

**Influence of Film Composition on Interfacial Enzyme Turnover**—Concerning the minimal specific activity of lipase as a function of molar fractions of triglyceride and lecithin (Fig. 5), we must first recall that minimal specific activity reflects enzyme turnover at the interface (6). Invoking the general model proposed by Verger et al. (3), we may imagine that the penetrated enzyme (E*) binds one substrate molecule to give the complex (E*S). This is the two-dimensional equivalent of the conventional Michaelis-Menten equilibrium. Once E* S is formed, catalysis then occurs, regenerating enzyme in the form of E*, accompanied by liberation of reaction products. It can be expected that changing the interface lipid composition and thus interfacial quality will affect minimal specific activity, which is determined by at least three parameters (3): substrate concentration (molecules/cm'), the two-dimensional Michaelis-Menten constant (K*,*) and the catalytic rate constant (kcat).

On one hand, we may say that a decreased substrate concentration could explain a decrease, but not an increase, in minimal specific activity (cf. Fig. 5). On the other hand, we may envision that a modified interfacial quality brought about by substrate dilution could increase kcat and/or decrease K*,* thus explaining the positive effect on minimal specific activity.

Adamich and Dennis (33, 34) recently reported that the specificity of phospholipase A2 in mixed Triton/phospholipid micelles could be reversed when more than one lipid was present. This reversal of apparent substrate specificity was explained as either a lipid-lipid interaction leading to a conformational change at the lipid-water interface or as a result of the "dual phospholipid" model of Roberts et al. (35). In addition to these attractive hypotheses, the present results can be extrapolated to more complex lipid mixtures, in which each lipid substrate could have a variable affinity for the enzyme at the interface. Thus, the enzyme can bind and catalyze each class of lipid molecules very differently as a function of the overall quality of the interface and independently of associations with the interface. This may be compared to the effects of solvent properties on enzyme activities in conventional aqueous enzymology.

Complex effects have been observed during phospholipase
lipase is best adapted for hydrolysis of mixed triglyceride.

Lipase turnover number on mixed trioctanoylglycerol/didodecanoylphosphatidylcholine films was increased 3 to 4 times by colipase. Apart from the 70% increase in enzyme activity observed with tributyrin emulsions (37), this is the greatest reported stimulatory effect of colipase on lipase turnover number. This activation was not attributed to protection from surface denaturation (8), since kinetics under these conditions were linear for considerable periods of time. In addition, a mixed film containing 35% phosphatidylcholine was hydrolyzed three times more rapidly than a pure triglyceride film. This positive effect could either be ascribed to a better hydrolysis of “natural” lipid mixtures, e.g., erythrocyte and ghost membranes (2, 34, 36). In particular, a reversal of lipid composition (34). A forthcoming publication from our laboratory will show that lipid packing can also have a great specific stimulation, a mixed film containing 35% phosphatidylcholine was increased 3 times. In this case, the active site of pancreatic lipase, which is known to be involved in the kinetic mechanism of lipolysis (38), could be more efficiently exposed to the substrate.

No evidence of phospholipid hydrolysis in mixed films of trioctanoylglycerol/didodecanoylphosphatidylcholine was found.

REFERENCES


2 No evidence of phospholipid hydrolysis in mixed films of trioctanoylglycerol/didodecanoylphosphatidylcholine was found.

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